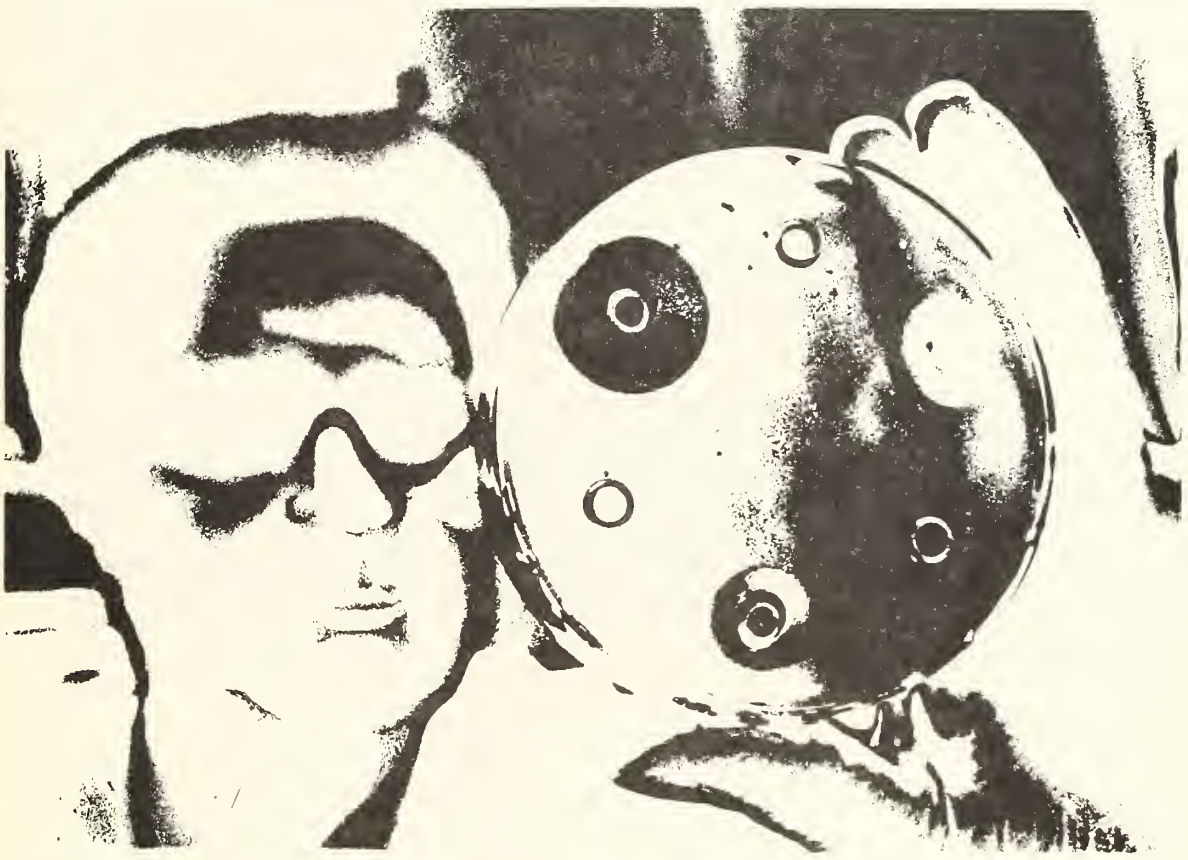
MICROBIOLOGY LABORATORY GUIDEBOOK



Scientific Services • Food Safety And Quality Service
U.S. DEPARTMENT of AGRICULTURE • Washington, D.C. 20250

FOREWORD

The microbiology laboratories of the Federal Meat and Poultry Inspection Program use the methods in this Guidebook unless they are, for some reason, inapplicable.

The looseleaf form makes minor revisions simple. Keep the numbered memorandum accompanying each revision to be sure that you have missed none. Fill out and return the form below each memorandum to receive the next revision. To obtain a reasonable number of additional copies, write to Microbiology Staff, Scientific Services, Meat and Poultry Inspection Program, U.S. Department of Agriculture, Washington, D.C. 20250.

Effective May 8, 1977, the functions performed by the Meat and Poultry Inspection Program were transferred from the Animal and Plant Health Inspection Service to the Food Safety and Quality Service.

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MICROBIOLOGY LABORATORY GUIDEBOOK

1. INTRODUCTION

Suggestions for revision of the Microbiology Laboratory Guidebook (MLG) are welcome. The MLG is distributed in looseleaf form, for easy revision. Each revision will be distributed with a numbered memorandum. Recipients should save all such memoranda so that any missing revisions will be discovered. Each time a revision is distributed, recipients must return the form at the bottom of the memorandum to receive the revision that will follow. Copies of the MLG are available from Microbiology Staff, Scientific Services, Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Washington, D.C. 20250.

1.1 HANDLING SAMPLES AND RECORDS

Study all records and correspondence before examining the sample. Maintain and handle the sample to insure that it is the same one that was collected, that it has not been tampered with, and that its condition is the same as it was at collection. Store reserve portions to maintain integrity for repeat analysis.

See detailed sampling and shipping instructions in the Manual of Meat Inspection Procedures, section 318.97 and in the Poultry Inspectors Handbook, pages 109-112.

2. ORGANOLEPTIC EXAMINATION

The term "organoleptic" refers to the use of the senses in determining the fitness of a product. It is important that the analyst have a thorough knowledge of the physical characteristics of both the normal product and the abnormal product. This knowledge can be gained by either experience or by special training.

When the question to be answered is related to spoilage, the organoleptic results are of primary importance; chemical and/or bacteriological results are corroborative and substantiating.

Examine every sample, or an appropriate portion of every sample, for appearance and odor. In some instances, use a smell panel. Note any abnormality on the laboratory report.

By Bernard F. Surkiewicz, Microbiologist

3. EXAMINATION OF FRESH OR PREPARED FOODS

The procedure for determining aerobic plate count is the same as that published by the AOAC (3.8). The procedures for determining numbers of coliforms and Escherichia coli differ from the AOAC procedures as follows:

(1) Use a single tube of Lauryl Sulfate Trytose Broth (LST) per dilution, rather than 3-tubes.

(2) Incubate inoculated LST and EC broths 24 ± 2 hrs.

(3) Consider gassing LST and gassing EC broths positive for coliforms and E. coli respectively, with no further testing.

The procedure for determining the numbers of Staphylococcus aureus (3.6) differs from the AOAC procedure by requiring one tube of Trypticase Soy Broth (containing 10% NaCl) per dilution, rather than 3 tubes.

These methods are applicable to the routine bacteriological examination of samples collected in federally inspected meat and poultry establishments producing fresh, frozen, cooked, smoked, or dehydrated meats and poultry; refrigerated salads; prepared or pre-cooked products (such as pot pies, lunch meats, dinners, breaded cutlets, dehydrated soups, etc.); and the ingredients incorporated with meat and poultry products (such as spices, vegetables, breading material, milk powder, dried egg, etc.).

Do not composite components of items such as frozen dinners into a single sample. Examine as separate samples the vegetable servings and meat servings deposited in separate compartments of the same tray.

Use the AOAC procedures (3.8) for determining numbers of coliforms, E. coli and S. aureus in samples of products under consideration for regulatory action.

The alternate method (direct plate count) for the isolation of S. aureus (3.7) is suitable for the analysis of food samples such as those implicated in staphylococcal food poisoning, in which large numbers of S. aureus may be expected. It is more rapid than that described in 3.6.

3.1 EQUIPMENT AND MATERIALS

(1) Quebec Colony Counter and Tally Register
(2) Balance, Capacity 2 Kilo, Sensitivity 0.1 g.
(3) Blender and Sterile Blender Jars
(4) Sterile Forceps, Spoon, etc.
(5) Sterile 10 ml., and 1 or 5 ml. Pipettes
(6) Incubator at 35 ± 1° C.
(7) Water Bath at 45.5 ± 0.05° C.
(8) Water Bath at 37 ± 1° C.
(9) Simple Stain Reagents
(10) Standard Petri Dishes, Glass or Plastic, Sterile
(11) Desiccated Coagulase Plasma, Rabbit
(12) Transfer Loop, 3 mm.
(13) Microscope
(14) Butterfield's Phosphate Diluent (20.1)

3.2 MEDIA

Plate Count Agar (20.2); Lauryl Sulfate Tryptose (LST) Broth (20.3); EC Broth (20.4); Baird-Parker Medium (20.5); Brain Heart Infusion (BHI) Broth (20.6); Tryticase Soy Broth with 10% Sodium Chloride (20.7).

3.3 PREPARATION AND DILUTION OF THE FOOD HOMOGENATE

Whenever convenient, weigh portions of frozen samples without thawing to avoid the effect on microorganisms of thawing and re-freezing, if a re-examination is necessary; otherwise, partially thaw at 2-5° C. for ca. 18 hours or by immersing water-tight sample containers in cold water 1-2 hours.

Using sterile spoons, forceps, scissors, etc., weigh aseptically 50 ± 0.1 g. of the sample into a sterile blender jar. Add 450 g. sterile diluent (20.1) and blend 2 minutes, to give 40,000 to 50,000 revolutions. This is the 10^{-1} dilution. Not more than 15 minutes should elapse from time sample is blended until all dilutions are in appropriate media.

If sample consists of less than 50 g., weigh about half the sample, and add the amount of diluent required to make the 1:10 dilution (nine times the weight of the portion of sample used). The total volume in the blender jar must completely cover the blades.

Permit foams to settle; then pipet 10 ml. of the blended 10^{-1} dilution into a 90 ml. dilution blank for the 10^{-2} dilution. Repeat to prepare dilutions of 10^{-3}, 10^{-4}, etc. Shake all dilutions 25 times in a one-foot arc. Use a separate 10 ml. pipette to prepare each dilution.

Pipettes must deliver accurately required volumes. Do not deliver less than 10% of their volume. For example, to deliver one ml., do not use a pipette of more than 10 ml. volume.

Hold reserve of samples in the freezer at or below 5° F. (unless the product is stored normally at ambient temperature) until determined that a repeat examination is not necessary.

3.4 AEROBIC PLATE COUNT

Pipet 1 ml. from 10^{-2}, 10^{-3}, 10^{-4}, etc., dilutions, using separate sterile pipettes for each dilution. Use additional dilutions when expecting higher bacterial levels. Place 1 ml. of the appropriate dilution into each of duplicate petri dishes, and add molten Plate Count Agar (cooled to 45 $\pm$ 1° C. in water bath). Mix by swirling or tilting plates to disperse the inoculum throughout the medium. Incubate 48 $\pm$ 2 hours at 35 $\pm$ 1°.* Using the Quebec Colony Counter, count duplicate plates in suitable range. If plates do not contain 30-300 colonies, record dilution counted and the number of colonies found. Average the counts obtained from duplicate plates and report aerobic plate count per gram. Report incubation temperature used.

3.5 COLIFORM GROUP AND ESCHERICHIA COLI

Pipet 1 ml. from the dilutions into a single Lauryl Sulfate Tryptose Broth (LST) tube per dilution, using separate pipettes for each dilution. Begin with the 10^{-1} dilution. Maximum dilutions of sample must be sufficiently high to yield a negative end point. Incubate 24 $\pm$ 2 hours at 35 $\pm$ 1° C. for gas formation as evidenced by displacement of liquid in insert tubes or by effervescence when tubes are shaken gently. Consider those dilutions of LST producing gas to contain coliforms, and report coliforms per gram according to the highest dilution of gassing LST. When a "skip" occurs, report by utilizing the "Phelps Index" (for example: If the 10^{-1}, 10^{-2}, and 10^{-4} dilutions produce gas but the 10^{-3} dilution tube is non-gassing, report "1,000 coliforms per gram.")

Transfer, using 3 mm. loop, from every gassing LST tube to EC Broth tubes. Incubate EC tubes 24 $\pm$ 2 hours at 45.5 $\pm$ 0.05° C. in a covered water bath. Submerge EC tubes in the bath so that the water level is above the highest level of medium in the tubes. Record every tube producing gas, as evidenced by displacement of liquid in insert tube or by effervescence when tubes are shaken gently. Report E. coli per gram according to the highest dilution of gassing EC, utilizing the "Phelps Index" when a "skip" occurs.

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* To determine both psychrophiles and mesophiles, incubate a duplicate set of plates at 20° C. for 4 or 5 days.

3.6 STAPHYLOCOCCUS AUREUS

Pipet 1 ml. from the dilutions into a single tube of Trypticase Soy Broth (containing 10% NaCl) per dilution, using separate pipettes for each dilution. Begin with the 10^{-1} dilution. Maximum dilutions of samples must be sufficiently high to yield a negative end point. Incubate tubes 48 $\pm$ 2 hours at 35° C.

Using a 3 mm. loop, transfer a loopful from each growth-positive tube and from the next higher dilution tube to previously prepared Baird-Parker medium (20.5). Streak in a manner to give well isolated colonies. Incubate plates for 48 hours at 35° C.

From each plate showing growth, pick two or more of each of the following convex, shiny black colony types: (1) with or without narrow, gray-white margin, surrounded by clear zone extending into opaque medium; (2) with or without narrow, gray-white margin, surrounded by clear zone with inner opaque zone; or (3) with or without gray-white margin, 1.0-1.5 mm. diameter. Inoculate a small amount of growth into 13 x 100 mm. tubes containing 0.2 ml. of BHI Broth, and incubate 18-24 hours at 35° C.

Just before use, reconstitute desiccated coagulase plasma (rabbit) with EDTA according to the manufacturer's directions. Add 0.5 ml. of reconstituted plasma to the BHI cultures and mix thoroughly. Place tubes in a 37° C. water bath and examine each hour from 1 through 4 hours for organized clot formation. Record results in accordance with 3.81. Test known positive and negative cultures simultaneously with the sample.

Record as coagulase-positive all tubes showing organized clots in 4 hours. Record coagulase reactions for each isolate by dilution. Report S. aureus per gram, calculated as the reciprocal of the highest positive dilution, using the "Phelps Index" when a "skip" occurs. (See 3.5).

3.7 ALTERNATE METHOD FOR STAPHYLOCOCCUS AUREUS

Pipet 0.1 ml. from each dilution on a single plate of previously prepared Baird-Parker medium, using separate pipettes for each dilution. Begin with the 10^{-2} dilution (i.e., 0.1 ml. of the initial 1:10 blend). Maximum dilutions of the sample must be sufficiently high to yield a countable plate.

Distribute the inoculum evenly over the surface of the plate using a sterile, fire polished, bent-glass rod ("hockey stick") for each plate. Incubate plates for 48 hours at 35° C.

Select a plate at the dilution containing 30 to 300 colonies, if typical S. aureus colonies are among them. Count the number of colonies with the

characteristics described in Section 3.6. Pick 10 of the colonies counted and inoculate each into separate 13 x 100 mm. tubes containing 0.2 ml. of BHI Broth; emulsify thoroughly. Pick all characteristic colonies if fewer than 10 are present on the countable plate.

Test for coagulase as in 3.6.

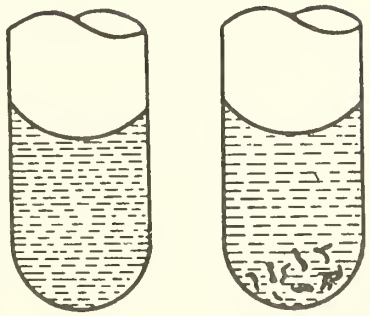
Calculate the total number of colonies represented by coagulase positive cultures and multiply by the appropriate sample dilution factor to record the number of S. aureus per gram.

3.8 REFERENCES

(1) Association of Official Analytical Chemists, 1970. Official Methods of Analysis of the AOAC. Eleventh Edition, pp. 842-844 (41.013 to 41.018)

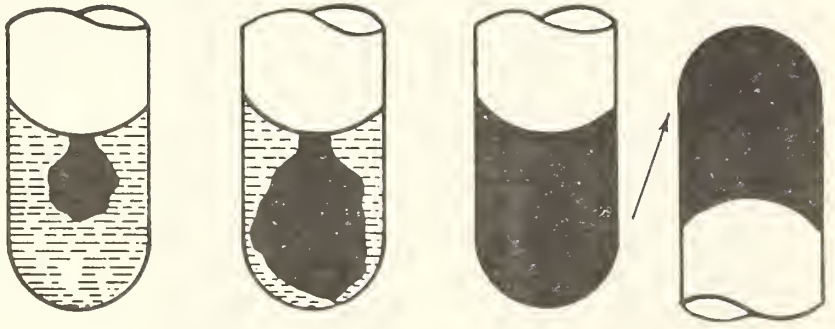
(2) Association of Official Analytical Chemists, 1970. Microbiological Methods. Official First Action. J. Assoc. Official Analytical Chemists, 54, No. 2, pp. 495-496

TYPES OF COAGULASE TEST REACTIONS



NEGATIVE

[no organized clots]



POSITIVE

[organized clots]

Prepared by Alice B. Moran, Microbiologist

4.0 ISOLATION AND IDENTIFICATION OF SALMONELLA FROM FOODS.

The procedures herein described have found wide acceptance in the United States and are in general use. The procedure for culturing dried milk was developed by the late Mr. W. R. North, Food and Drug Administration. The procedure for culturing dried eggs was developed by Mr. North and Dr. M. T. Bartram. This method was adapted by other workers in the Food and Drug Administration to other dried products and to frozen foods. The procedure for culturing raw meat is an adaptation of Dr. F. Kauffmann's "combined enrichment method" with modifications of Hajna and Damon and the late Mrs. Mildred Galton.

4.1 EQUIPMENT, REAGENTS, MEDIA

4.11 Equipment

(1) Sterile pint and two-quart Mason jars with screw-on lids.
(2) Sterile tablespoons, scissors, forceps, knives, glass stirring rods, pipettes, petri dishes.
(3) Sterile blender jars, or Osterizer blender with sterilized cutting assemblies and adapters for use with Mason jars.
(4) Tergitol 7 (sodium heptadecyl sulfate), steamed 1/2 hour.
(5) Whirl-Pak bags.
(6) Incubator, 35° ± 1° C.
(7) Water bath, 48° ± 1° C.
(8) Glass slides or glass plate marked off in one-inch squares.

4.12 Reagents

(1) Brilliant green dye 1% Aqueous solution, steamed.
(2) Crystal violet dye, 1% Aqueous solution, steamed.
(3) Iodine solutions.
(4) Cystine, 1% Aqueous solution, filter sterilized.
(5) Methyl red reagent.
(6) O'Meara's V-P reagent, modified (20.38).
(7) Kovac's reagent. Refrigerate.
(8) Ferric chloride, 10% Aqueous solution.
(9) KCN.
(10) Urea.
(11) Sodium deoxycholate, 10% Aqueous solution, sterile.
(12) Sterile mineral oil.
(13) Saline, 0.85%.
(14) Saline, 0.85% with 0.6% formalin.
(15) Sodium hydroxide, IN.

4.12 (Con.)

(16) Salmonella polyvalent O serum.
(17) Salmonella polyvalent H serum.
(18) Salmonella O grouping serum.
(19) Carbohydrates: glucose, lactose, sucrose, salicin, dulcitol, adonitol, inositol, sorbitol, arabinose, raffinose, rhamnose, xylose, trehalose, maltose.
(20) Amino Acids: l-lysine, l-arginine, l-ornithine.

4.13 Media

Lactose Broth (20.15); Tetrathionate Broth (20.16); TT Broth (20.17); Selenite-Cystine Broth (20.18); Brilliant Green Agar (20.19); Brilliant Green Sulfa Agar (20.20); XL Agar (20.21); XLD Agar (20.21); SS Agar (20.22); Bismuth Sulfite Agar (20.23); TSI Agar (20.24); Lysine Iron Agar (20.25); MR-VP Medium (20.26); Tryptone Water (20.27); Simmons Citrate Agar (20.28); Phenol Red Tartrate Agar (20.29); Motility Medium (20.30); Urea Agar (20.31); Fermentation Broth (20.32); Decarboxylase Medium (20.33); Malonate Broth (20.34); KCN Medium (20.35); Phenylalanine Agar (20.36); Nutrient Gelatin (20.37); Trypticase soy broth (20.39); Tryptose broth (20.40); Veal infusion broth (20.41); Lactose broth, 10X (20.42); Trypticase soy agar (20.53).

4.2 GENERAL DISCUSSION OF MEDIA AND PROCEDURES

(1) Weighing samples. Samples should be weighed in a hood so that disinfectant fogging devices can be used to settle dust, and the environment in the vicinity of the balance can be better controlled. At the very least, the balance should be kept in an area that is protected from strong air currents. Before starting, wipe off the workbench area and the balance with a non-corrosive disinfectant in water solution. Leave the surface moist, and as it dries, renew the disinfectant. Many of the materials to be weighed are fine, dry powders and every precaution must be taken to prevent this matter from contaminating the laboratory environment.

All instruments used in taking the samples must have been terminally sterilized either in an autoclave or sterilizing oven. If necessary when working on unusually large numbers of samples and when instruments are in short supply, they may be washed with soap and water, rinsed, and steamed for thirty minutes in an instrument sterilizer (Caution: Scissors must not have any organic matter in the hinges.)

Do not dip instruments into alcohol and flame as a substitute for heat sterilization. It is not as certain a method of sterilization as in steam. Since the information obtained in this work may become the basis for regulatory action, there can be no excuse for using methods that are in any way questionable.

4.2 (Con. #1)

Weigh the product into sterile containers. When weighing is complete, clean and disinfect the area. Never touch the product with your hands. Keep in mind that methods used for recovery of Salmonella species have been designed to detect very small numbers. Positive isolations must come from the food samples and not from the laboratory environment as a result of poor bacteriological technique.

(2) Enrichment. In almost every food product examined, members of the genus Salmonella will comprise but a small percentage of the total microbial population. Therefore, the methods that will be used to recover organisms are those that have been devised to enhance their growth while keeping the growth of competitive organisms at a minimum. Selective enrichment methods are not required when culturing materials that have been protected from gross contamination (shell eggs, for example), but such materials have been encountered rarely by the Scientific Services Staff. With each material examined, therefore, one or more of the selective enrichment media will be used.

Two very popular enrichment media are Tetrathionate Broth (4.9(3)), or one of its many modifications, and Selenite F Broth (4.9 (5)), both in its original form and as modified by North and Bartram (4.9 (20)). There are other enrichment media, no doubt excellent, that are less widely used. Basically, most enrichment media were originally designed to isolate salmonellae from feces, and this is a very different proposition indeed from isolation from food. As a matter of fact, each type of food to be examined presents its own problems, and after a general introduction to the subject of isolation, each type of food will be dealt with separately in this section of the manual.

Commercial Tetrathionate Broth is a peptone medium to which is added bile salts to inhibit the growth of gram-positive organisms, brilliant green to inhibit the growth of gram-positive and the gram-negative lactose-fermenting organisms, and a concentration of tetrathionate that, if not harmless to salmonellae, is at least less toxic to them than to other enteric bacteria. The medium is highly buffered by calcium carbonate and therefore, pH is not a matter of much concern during the incubation process.

Tetrathionate Broth of Mueller (4.9 (19)), as modified by Kauffman (4.9 (27)), has in turn been modified by many workers. TT Broth (4.9 (4)), is a modification of Kauffmann's medium and has been greatly enriched with yeast extract.

Selenite F Broth (4.9 (5)), also has a peptone base. It employs sodium selenite to inhibit the growth of other enteric organisms and, in the North and Bartram modification, cystine to enhance the growth of

4.2 (Con. #2)

salmonellae in the presence of large amounts of organic material. The medium also contains lactose to provide a carbohydrate that, when fermented, will produce acid. The enrichment effect of Selenite F Broth, which has been studied by Leistner, et al (4.9 (21)), depends on an acid pH: If the pH goes below 5.8, all Enterobacteriaceae, are suppressed. Between 5.8 and 6.3, salmonellae are selectively favored. Above pH 6.3 other Enterobacteriaceae, particularly Proteus and Enterobacter, may overwhelm Salmonella.

Raw meat and other animal products that have been mishandled and are undergoing spoilage, are added directly to selective enrichment media. Normally raw meat and products wherein bacteria have been subjected to freezing, drying or chemical treatment, and are presumably in an attenuated state, are given a preliminary treatment. Some dry products such as milk are merely reconstituted with water, the product itself thereby becoming the culture medium. In other cases, the products are rehydrated in an unselective nutrient medium. Lactose Broth (4.9 (2)), has been used successfully in many laboratories for this pre-enrichment step. Lactose is not a sugar that is usually fermented by Salmonella, but the fermentation of lactose by competitor organisms lowers the pH of the medium. It is said that the effectiveness of lactose in the pre-enrichment is due to this fact, because Salmonella is less affected by a lower pH than are certain other organisms. A portion of the lactose broth culture is cultured further in selective enrichment media. It might be expected that organisms able to ferment lactose would be multiplying rapidly and would therefore succumb more quickly to the lethal effect of chemicals in the selective enrichment medium. It is a fact that actively growing cells are more susceptible to the bactericidal effect of chemicals.

(3) Plating. While selective enrichment media give salmonellae an opportunity to grow, the resulting culture usually has a highly mixed flora. Therefore, many plating media have been devised to make easier the selection of colonies of salmonellae from among those of other organisms. In principle these consist of a basic nutrient medium with inhibitors to suppress the growth of unwanted organisms and an indicator system to reveal, by characteristic color, colonies likely to be Salmonella.

Many highly selective plating media have been devised and a variety are available from commercial sources. All of them depend on recognition of salmonellae by one of two characteristics: (a) by production of H_2S and (b) by a carbohydrate fermentation pattern--most commonly failure to ferment lactose or lactose and sucrose.

4.2 (Con. #3)

While most members of the genus Salmonella produce H_2S, some do not. So this one characteristic cannot be depended on for the selection of all salmonellae. At the same time, aberrant Salmonella cultures are known that ferment lactose. These are atypical in appearance on those media that use lactose for a differential sugar. Obviously, the most thorough job of isolating the organisms can be done when two media, one of each type, is used.

Since highly selective media tend to be inhibitory also for salmonellae, usually one less selective medium is also employed. The choice of media is governed by the flora likely to be found in the product examined. The total number of media is limited by practical considerations.

The surface of agar plates must be dry before they are used. Brilliant Green Agar (4.9 (3)), SS Agar (4.9 (8)), and the bismuth sulfite medium of Wilson and Blair (4.9 (22)), are among the most widely used plating media for isolation of salmonellae from materials that have been cultured in enrichment broth. MacConkey Agar (4.9 (23)), is less inhibitory than these media and for that reason less useful for plating enrichment broth, where the flora is likely to be highly mixed and may contain swarming Proteus. Nevertheless, there may be times when a relatively non-inhibitory medium is wanted. A medium developed in recent years that is proving to be very useful, is XL Agar of Taylor (4.9 (7)). Since judgment in the selection of colonies depends on an understanding of the composition of the medium and how it functions, a brief discussion of each of the above media follows.

Brilliant Green Agar (BGA) is basically a peptone-yeast-extract medium with brilliant green added to it to suppress the growth of gram-positive organisms and lactose-fermenting, gram-negative organisms. It may also have sulfapyridine added (BGS) to suppress Proteus (4.9 (6)). Its selectivity system consists of two fermentable carbohydrates, lactose and sucrose, and, as an acid indicator, phenol red. Since salmonellae are unable to ferment either lactose or sucrose, and since their metabolism of peptone results in alkaline end-products, colonies of Salmonella are pink and are surrounded by a reddened medium.

On the other hand, any organism that can ferment lactose or sucrose or both, produces colonies that are green or yellow and are surrounded by a yellowish-green medium. On plates where there are few salmonellae, and these few are near colonies that are green or yellow, a colony of Salmonella sp. may have a brownish appearance without any reddening of the surrounding medium.

4.2 (Con. #4)

SS Agar has a beef extract and peptone base and contains bile salts and brilliant green as inhibitors of gram positive and lactose-fermenting, gram-negative organisms. It has two selectivity systems. Ferric and sodium citrates and sodium thiosulfate make up the H_2S indicator system; lactose and neutral red make up the fermentation-indicator system. Organisms that ferment lactose, and therefore produce acid, are pink in color, while salmonellae and other organisms that either fail to attack lactose, or do so very slowly, give uncolored colonies. Sucrose may be added to the medium to enhance its differential value if sucrose-fermenting organisms are a problem. Black-centered colonies sometimes will develop on this medium when cultures produce hydrogen sulfide.

MacConkey Agar is less inhibitory than brilliant green and SS Agar. Again there is a basic peptone medium with a small amount of bile salts and crystal violet to inhibit gram-positive organisms. The selectivity system has lactose as a fermentable carbohydrate with neutral red as an acid indicator. Lactose-fermenting organisms appear as red colonies against a cloudy background of precipitated bile, while salmonellae are transparent and without color. If desired, sucrose can be added to differentiate sucrose-fermenting organisms.

The reasoning behind the development of the plating media so far discussed is based on the fact that Salmonella cultures fail to ferment lactose or sucrose and therefore any lactose or sucrose-fermenting organisms can be avoided in picking colonies for further screening. Whereas this is true as far as recognition of the common Salmonella serotypes is concerned (types in sub-genus I in Kauffmann's (4.9 (26)) terminology), it would be an error to assume that fermentation of lactose can be associated with non-pathogenicity. There are occasional aberrant cultures of the common Salmonella serotypes that ferment lactose but more important, there is a large group of organisms very closely related to Salmonella that ferment lactose.

Most of these are slow and reluctant lactose-fermenters, but others ferment lactose rapidly and cannot be distinguished from coliform organisms on those plating media that depend on any indicator system involving production of acid from lactose. This group of organisms is known as the "Arizona Group" in the U.S., and in veterinary literature the term "Arizona paracolon" is common. In Bergey's Manual (4.9 (24)), the name is "Paracolobactrum arizona", but this name has not gained wide acceptance. Dr. W. H. Ewing (4.9 (25)), has proposed the name "Arizona hinshawii." F. Kauffmann proposes to place the organisms in subgenus III of the genus Salmonella with the name Salmonella arizona. While awaiting final disposal of the question, this manual will adopt Kauffmann's proposal and refer to members of the group thus: Salmonella sp., subgenus III (Arizona).

4.2 (Con. #5)

It happens that the two serotypes in the group that are common in turkeys are both slow lactose-fermenters and therefore appear on SS and Brilliant Green Agar just like Salmonella, subgenus I. However, in order not to miss the types that ferment lactose rapidly, another type of medium plating is used in which the indicator system is not based on any attack on carbohydrates but is based on detection of H_2S production.

Bismuth Sulfite Agar (Wilson and Blair) does not depend on any fermentative action of the colony for recognition of Salmonella sp., because glucose, which is the medium's fermentable sugar, if fermented by all Enterobacteriacae. Most Salmonellae of sug-genera I and III produce abundant H_2S and appear on this medium as black colonies surrounded by a brownish-black zone exhibiting a metallic sheen. Plates of this medium should be prepared at least 48 hours before they are used.

XL Agar, developed by Taylor for selection of shigellae, is also very useful for selection of salmonellae. In addition to its H_2S indicator system, it contains[13] three fermentable sugars: lactose, sucrose and xylose. After salmonellae exhaust the xylose in the medium, they decarboxylate lysine, causing a reversion to an alkaline pH and a red color with the phenol red indicator. Lysine-positive coliforms do not revert to red colonies because the lactose and sucrose in the medium produce acid in excess. XL Agar plates often require a full 24 hours to develop black-centered colonies. After one day's incubation, salmonellae plated from enrichment broth are typically yellow with black centers; in 48-hours they turn from yellow to pink. Only when pure cultures are plated, is the pink color seen after one day's incubation. Sodium deoxycholate may be added (XLD) to prevent swarming of proteus.

Plates are incubated at 35° C. The brilliant green agars, SS, MacConkey and the XL agars are examined after overnight or 24-hours incubation and returned to the incubator if no suspicious colonies have appeared. The plates should be re-examined after 48 hours and at that time the Bismuth Sulfite plates are examined.

Colonies typical of Salmonella sp. are marked by circling the plate with marking pens. Ordinarily, a total of three colonies are selected when many typical colonies are found, but in doubtful cases three or four from each plate may be selected for inoculation into screening media. Plates are then reserved in case it is necessary to go back to them.

(4) Rapid screening. For rapid screening, it is convenient to use two media in tandem: Triple Sugar Iron Agar (4.9(9)). (TSI), and Lysine Iron Agar (4.9(10)), slants: TSI contains a small amount of

4.2 (Con. #6)

glucose and larger amounts of sucrose and lactose and it has an H_2S indicator. The medium is yellow when acid and red when alkaline. The amount of glucose present is sufficient, when fermented, to cause an acid reaction in the butt; but on the surface this acid is oxidized, and aerobic attack on the peptone results in alkaline end products, and hence the slant turns red. If either lactose or sucrose is fermented, the slant will also be acid, since there will be more acid formed than can be oxidized. Accordingly, if the entire slant is yellow, either lactose or sucrose or both have been fermented. If the butt is yellow and the slant red, then glucose only was fermented; salmonellae give this reaction. If the entire slant is red, none of the sugars were fermented and such a culture cannot belong in the family Enterobacteriaceae. The presence of H_2S blackens the medium. Lysine Iron Agar contains a small amount of glucose, an H_2S indicator and the amino acid lysine. All of the Enterobacteriaceae will produce enough acid from the glucose to turn the butt, but not the slant, yellow. The decarboxylase enzyme removes CO_2 from the amino acid forming an amine and hence an alkaline (purple) reaction. Lysine decarboxylase-positive cultures are therefore purple. Negative cultures have yellow butts. H_2S causes slight blackening.

The reactions of the most common Salmonella types are given below. Also given are some reactions that are less typical. In selecting cultures for further study, and in the absence of the common types, keep the less common types in mind, and do further biochemical tests on any organism that is both H_2S and lysine positive (2) and check with Salmonella O sera those that react like 3 and 4 below.

		TSI Agar (24 hrs.) Butt	Slant	H_2S	LI Agar (24 hrs.) Butt	H_2S
1.	Common Salmonella in Subgenera I and III	Y	R	+	P	+
2.	Certain members of Subgenus III	Y	Y	+	P	+
3.	S. pullorum S. gallinarum and certain others	Y	R or trace	-	P	-
4.	S. typhi-suis	Y	R	-	Y	-

Y = Yellow R = Red P = Purple

4.2 (Con. #7)

The tests needed to orient a culture within the family Enterobacteriaceae (to confirm it as Salmonella or assign it to another genus) are production of indol, methyl red reaction, production of acetyl-methyl-carbinol from glucose (Voges-Proskauer test), growth on citrate medium, hydrolysis of urea, liquefaction of gelatin, ability to grow in KCN, presence of the enzymes lysine and ornithine decarboxylase, arginine dihydrolase, phenylalanine deaminase, ortho nitrophenyl-B-galactosidase, motility, gas production from glucose fermentation, and production of acid from dulcitol, salicin, mannitol, lactose, sucrose, maltose, adonitol, sorbitol, inositol, arabinose, raffinose, rhammose, trehalose, malonate, mucate and d-tartrate. Not all these tests will be done on each culture. In fact, the minimum number needed to establish the culture as Salmonella is all that need be done.

If serology is to be done, ideally it should follow, not precede, the completion of biochemical testing. However, the procedure that this manual offers permits of short-cuts. The serological procedures outlined are the minimum needed to confirm an organism as a Salmonella and are not to be confused with Salmonella serotyping.

(5) Evaluation of Plating Media. The work of Read and Reyes (4.9(30)) calls attention to the variability in efficiency of brilliant green agar for isolation of salmonellae. This variability applies also to all of the more inhibitory selective plating media. Therefore, it is necessary to evaluate plating media to make sure that any medium is not unduly inhibitory to the organism it is designed to isolate. Since this is time-consuming, it is wise to purchase a large supply from a single lot. Make arrangements with the manufacturer to supply a test bottle, with the option of buying a large supply of the same lot number if the test bottle proves to be satisfactory.

The following procedure for testing media makes use of one common serotype that grows easily and four additional serotypes that are animal pathogens, and that grow much more poorly on isolation media.

A medium is unsatisfactory if its efficiency in growing Salmonella typhi-murium is less than 75% as compared with trypticase soy agar (20.51). The four additional strains, S. cholerae-suis, S. typhi-suis, S. pullorum and S. gallinarum, do not figure in the efficiency rating but serve to orient the worker in regard to what can be expected from the more fastidious organisms on the medium studied.

Inoculate 5 tubes of trypticase soy broth with S. typhi-murium, S. cholerae-suis, S. typhi-suis, S. gallinarum, and S. pullorum. Incubate 18 hours.

4.2 (Con. #8)

Make dilutions of a culture in Butterfield's buffered diluent (20.1). Dilute S. typhi-murium, S. cholerae-suis, S. gallinarum, and S. pullorum 10^{-5}, 10^{-6}, 10^{-7}. Dilute S. typhi-suis 10^{-4}, 10^{-5}, and 10^{-6}. For each dilution of each culture use three dried plates of test medium and three dried plates of trypticase soy agar (20.53). Use exactly 0.1 ml. of culture dilution to inoculate each plate. Spread the inoculum with sterile glass hockey sticks. Complete the plating within fifteen minutes after a dilution is made.

The dilutions suggested usually result in at least one countable set of plates. If they do not, the test must be repeated on a new 18-hour culture.

Incubate 2 days, then count the colonies and also measure the colony size.

$$\% \text{ efficiency} = \frac{\text{no. of colonies on plating medium}}{\text{no. of colonies on trypticase soy agar}}$$

Test all Salmonella plating media in this manner.

4.3 ISOLATION PROCEDURES

4.305 Sample Pooling

When examining products that reasonably can be expected to be Salmonella-free, valuable time, labor, and materials can be saved by sample pooling. There are several ways this can be done.

Pooling at the non-selective enrichment step (lactose broth) is appropriate when the likelihood of finding salmonellae is nil or when, if a positive is found, it is not important to know which particular twenty-five gram sample contained the organism.

(a) Those samples that are to be examined only for salmonellae may be pooled in the blenders. Up to thirty samples may be combined, depending, of course, on the capacities of the blenders and culture flasks that are available. The proportions of sample volume to broth must be maintained. The lactose broth should be warmed in advance.

(b) When food homogenates (see 4.35) are pooled, the culture flask should be warmed in a water bath to bring the contents to 35° C. before transfer to the incubator.

(c) Incubation of large pools should be prolonged to two days. Subculture 10 ml. of the incubated lactose broth pool in 100 ml. of selective enrichment, and proceed as usual.

In cases in which it is important to be able to identify which particular samples may contain salmonellae, it is still possible to take advantage of labor-saving by pooling. In such cases, the samples may be started in the usual way in lactose broth. After incubation, up to ten of these cultures may be pooled in selective enrichment broth. The remaining broth (or a portion of it) is refrigerated. The total amount of selective enrichment broth used will be the same, but the number of plates to be streaked is reduced. If a positive pool is found, all the pooled samples are started individually in selective enrichment broth by going back to the refrigerated lactose broth.

4.31 Powdered eggs, breading mixes, dehydrated sauces, dried milk.

Use AOAC (4.9(29)), Paragraph 41.024 et seq.

4.32 Precooked Frozen Foods. (See also 4.305 Sample Pooling)

(1) Weigh 25 grams into sterile blender jar. Add 225 ml. sterile lactose broth (20.15). Blend 2 minutes. If sample contains fat, add Tergitol 7 (from 0.5 to 0.6%).

(2) Check pH with narrow range test paper by removing a drop with a sterile pipette. Adjust pH to neutrality with 1N NaOH using a sterile pipette.
(3) Incubate for a full 24 hours at 35°.
(4) Transfer 0.5 ml. of incubated broth to 10 ml. TT (20.17) and Selenite-cystine broth (20.18).
(5) Incubate 35° C. for 18-24-hours.*
(6) Streak above enrichments on BGS (20.20) and XLD (20.21) Agars.
(7) Incubate 35° C.
(8) Examine in 22-24 hours. Reincubate negative plates and re-examine the following day.
(9) Select colonies - see 4.4 et seq.

4.33 Salted Natural Casings.

(1) Prepare a series of jars of sterile saline solutions as follows: 200 ml. each of 15%, 10%, 5% and 0.85% NaCl.
(2) Weigh 25 grams of product into the 15% saline. Twirl until all salt is washed off sample. Leave ten minutes. Pour saline into a sterile Seitz filter. Transfer sample successively to 10%, 5%, and 0.85% saline with ten minutes soaking in each. Pour saline solutions successively through the filter ending with the 0.85% saline.

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* The analyst may, at his discretion, incubate the enrichment media at 43° C. in a carefully controlled waterbath, instead of at 35° C. This alternative is being evaluated, and may be recommended in future revisions.

(3) Mince the washed sample with sterile scissors. After all the washings have been filtered, remove the filter pad aseptically and add to the sample. Add 225 ml. sterile lactose broth (20.15). Shake.
(4) Incubate 35° C. for a full 24 hours. If there is no growth or only sparse growth in 24 hours, reincubate for an additional 24 hours.
(5) Transfer 0.5 ml. to 10 ml. TT broth (20.17).
(6) Incubate 35° C. for 18-24 hours.*
(7) Streak on BGS (20.20) and XLD (20.21) agars.
(8) Incubate 35° C.
(9) Examine in 22-24 hours. Reincubate negative plates and re-examine the following day.
(10) Select colonies - see 4.4 et seq.

4.34 Raw meat.

Ordinarily inoculate raw meat directly into lactose broth (20.15).

If the sample is not ground already it may be best to mince it with scissors (e.g. chunk meat) or leave it whole (e.g. chicken wings) to avoid jamming of blender blades with skin or connective tissue. Whirl-Pak bags can be used in culturing these samples. Often the culturing can be done in the sampling collection bag provided there are no punctures in the plastic.

(1) Weigh 25 grams of meat. Weigh ground meat into a sterile blender jar; other samples into a sterile jar or Whirl-Pak bag.
(2) Add 225 ml. lactose broth (20.15). Blend two minutes or shake thoroughly.
(3) Add Tergitol 7 at about the 0.6% level. Blend ground samples ten additional seconds or shake other samples twenty-five times. Do not remove whole meat pieces.
(4) Incubate 35° C. 18-24 hours.
(5) Transfer 0.5 ml. to 10 ml. TT broth (20.17).
(6) Incubate 35° C. for 18-24 hours.*
(7) Streak on BGS (20.20) and XLD (20.21) agars.
(8) Incubate 35° C.
(9) Select colonies. See 4.4 et seq.

4.35 Food Homogenate.

To isolate Salmonella from food samples homogenized as outlined in Section 3.3, use the 10^{-1} food homogenate. (See also 4.305 Sample Pooling).

* The analyst may, at his discretion, incubate the enrichment media at 43° C. in a carefully controlled waterbath, instead of at 35° C. This alternative is being evaluated, and may be recommended in future revisions.

4.35 (Con.)

(1) Weigh 250 grams of food homogenate into a sterile jar (this contains 25 grams of product).
(2) Add 26 ml. of 10 X lactose broth (20.42).
(3) Add Tergitol. Usually add 0.6%. If the product has a very large amount of fat, add up to 1%.
(4) Check pH with narrow range test paper by removing a drop with a sterile pipette. Adjust to pH 7.0 with 1 N NaOH using a sterile pipette. Shake.
(5) Incubate a full 24 hours at 35° C.
(6) Transfer 0.5 ml. to 10 ml. TT broth. (20.17)
(7) Incubate at 35° C. for 18 to 24 hours.*
(8) Streak on XLD (20.21 and BGS Agar (20.20).
(9) Incubate 22-24 hours, 35° C. Reincubate negative plates and re-examine the following day (see 4.4 et seq.).

4.4 EXAMINATION OF PLATING MEDIA

If plates have few typical colonies when first examined, return to incubator and re-examine later in day and the following day. Mark, for later picking, colonies that are well isolated.

(1) BG and BGS. Select colonies that are pink and opaque with a smooth appearance and entire edge surrounded by a red color in the medium. On very crowded plates, look for colonies that give a tan appearance against a green background.

(2) XL and XLD agars. Select colonies with black centers. The rim of the colony will be yellow in 24 hours; later it may turn red. (Yellow colonies with tiny black centers or with gray centers usually are Proteus sp.)

(3) SS Agar. Select colorless colonies that are opaque, transparent or translucent with or without black centers.

(4) Bismuth Sulfite Agar. After 48 hours incubation, select colonies that are black and are surrounded by a blackened medium that exhibits a metallic sheen by reflected light. Occasionally, colonies may be light green.

4.41 PICKING COLONIES FROM SELECTIVE MEDIA

Pick three colonies from a plate. Pick only from the surface and center of the colony. Avoid touching the agar, because these highly selective media suppress growth of many organisms that may nevertheless be viable.

4.41 (Con.)

If there are no well-isolated colonies on a plate, place a loopful of growth into a tube of TT or Selenite-Cystine medium and incubate overnight, then restreak to selective agars.

4.5 SCREENING MEDIA.

(1) Inoculation. Inoculate both Lysine Iron Agar (20.25) and TSI Agar (20.24) in tandem by stabbing the butts and streaking the slants in one operation. Loosen cap. Incubate at 35° for up to 24 hours.

(2) Examination. Examine TSI and Lysine Iron Agar slants as a set. Note the colors of butts and slants, blackening of the medium and presence of gas as indicated by gas pockets or cracking of the agar. Note also the appearance of the growth on the slants along the line of streak. Discard sets that show "swarming" away from the original site of inoculation. Discard sets that show a reddish slant in Lysine Iron Agar. Discard cultures not resembling Salmonella sp.

Confirm as Salmonella sp. by simple serological procedure those cultures that give an appearance typical of salmonellae.

Examine cultures suggestive, but not typical of Salmonella sp. by a combination of biochemical and serological procedures. Confirm as Salmonella sp. by biochemical tests those cultures that give an appearance typical of salmonellae but do not react serologically. Refer to the chart below for these procedures:

TSI Agar			Lysine Iron Agar		Sera			
Butt	Slant	H_2S	Butt	H_2S	Poly-O	O-Group	Poly-H	Disposal
								Record:
Y	R	+	P	+	+	+	+	Salmonella sp.
Y	R	+	P	+	+	+	-	Biochemical and Motility Tests
Y	R	+	P	+	+	-		Biochemical and Motility Tests
Y	R	-	P	-				Biochemical and Motility Tests
Y	R	-	Y	-	+	+	+	Biochemical and Motility Tests*
Y	R	-	Y	-	-	-	-	Discard
Y	R	+	Y	+or-				Discard
Y	Y	-	Y or P	-				Discard
Y	Y	+	P	+				Biochemical and** Motility Tests
NC	NC							Discard

* Salmonella typhi-suis found seldom in swine in U.S.
** Arizona Group; (Salmonella Subgenus III of Kauffmann).
NC No change in color over uninoculated medium.

4.6 BIOCHEMICAL PROCEDURES

Do the minimum number of tests needed either to discard the culture or establish that it is a member of the genus Salmonella.

Ordinarily, do not do exhaustive tests on any culture from a sample that has already yielded a typical, easily identifiable organism.

Inoculate the following media first: tryptone broth (20.27), MR-VP medium (20.26) (4.9(11)), Simmons Citrate Agar (20.28) (4.9(12)), Christensen's Urea Agar (20.31) (4.9(14)), motility medium (20.30) (4.9(1)), phenol red tartrate Agar (20.29) (4.9(13)), and glucose, lactose, sucrose, salicin and dulcitol fermentation broths (4.9(1)). Incubate at 35° or 37° C. and record appearance the following day. Test

4.6 (Con.)

tryptone water with Kovac's reagent (20.52) for indol production in 24 hours and, if negative, again in 48 hours. Do not do the MR-VP test until 48 hours have elapsed. If results are ambiguous, repeat MR test with five days' incubation. Hold carbohydrate fermentation tests for 14 days.

Refer to "Identification of Enterobacteriaceae", Edwards and Ewing, 3rd Edition, for biochemical reactions of Enterobacteriaceae (page 24), and for fermentation media and test procedures.

Discard all cultures that are either urea-or VP-positive.

Discard any culture that has the following combination of characteristics: produces gas in glucose, produces indol but not H_2S, is MR positive, VP negative and citrate negative. It is E. coli, regardless of whether it ferments lactose in 48 hours.

Inoculate further biochemical rests as necessary to eliminate other Enterobacteriaceae. Refer to Edwards and Ewing for details.

Eliminate Providencia sp. by a positive phenylalanine reaction.

Eliminate Enterobacter hafnia on the basis of the following biochemical pattern:

indol negative
MR negative and VP and citrate positive
based on four days incubation at 25° C.
Fermentation of arabinose and rhamnose
Failure to ferment adonitol, inositol, sorbitol and raffinose.

4.7 SEROLOGICAL TESTS

(1) O Agglutination tests. Make an opaque suspension of growth from TS1 or Lysine Iron Agar slants in one-half ml. of saline. On a glass slide, mix one loopful of it with a drop of Salmonella polyvalent serum. Mix a second loopful with saline. Tilt back and forth and watch for agglutination. Do not read agglutination tests with a hand lens. If there is agglutination with the saline alone, discard the culture. Identify such a culture by biochemical reactions. If the saline control does not agglutinate and the polyvalent serum does, test the culture with Salmonella O grouping antisera. Record positive results and proceed to H Agglutination tests.

4.7 (Con.)

(2) H Agglutination tests. Inoculate trypticase soy broth, tryptose broth or veal infusion broth. Incubate 35° C. overnight or until growth has an approximate density of three on McFarland's scale. Add an equal amount of saline containing 0.6% formalin and let stand one hour. Remove one ml. to an agglutination tube. Add Salmonella polyvalent H serum in an amount indicated by the serum titer. Incubate at 48° in a water bath for up to one hour. Record presence or absence of agglutination and discard tubes.

If desired, use Spicer-Edwards pooled serum or H typing serum. Find details in "Identification of Enterobacteriaceae," Edwards and Ewing, 3rd Edition (4.9(1)).

4.8 STORAGE OF CULTURES

Do not leave cultures on TSI Agar for a long period of time because this tends to cause roughness of O Antigens. Subculture Salmonella cultures by stabbing nutrient agar (0.75% Agar). Incubate 35° C. overnight. Seal with corks in hot paraffin wax.* Label cultures and store in the dark at room temperature. Do not store near a radiator. Such cultures will remain viable for several years.

4.9 REFERENCES

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	Common Salmonella sp. Subgenus I	S. typhi	S. paratyphi B	S. pullorum	S. gallinarum	S. typhi-suis	Salmonella sp. Subgenus II	Salmonella sp. Subgenus III (Arizona Group)	Salmonella sp. Subgenus IV
Indol	-	-	-	-	-	-	-	-	-
M R	+	+	+	+	+	+	+	+	+
V P	-	-	-	-	-	-	-	-	-
Citrate	+	-	+	-	slow +	-	+	+	+
Urea	-	usually -	-	-	-	-	-	-	-
H_2S	+	+	+	poor +	-	-	+	+	+
Motility	+	+	+	-	-	+	+	+	+
Glucose	+	A	+	+	A	+	+	+	+
Lactose	-	-	-	-	-	-	- or slow	+	-
Sucrose	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	slow +
Dulcitol	+	slow +	+	-	+	slow +	+	-	-
Tartrate	+	+	-	-	+	-	- or slow	- or slow	- or slow
Malonate	-	-	-	-	-	-	+	+	-
Lysine	+	+	+	+	+	-	+	+	+
Arginine	slow +	slow +	slow +	slow +	slow +	slow +	slow +	slow +	slow +
Ornithine	+	-	+	+	-	+	+	+	+
K C N	-	-	-	-	-	-	-	-	+
Gelatin	-	-	-	-	-	-	+	+	+

By Ralph W. Johnston, Senior Staff Officer,
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5.1 THE EXAMINATION OF HEAT PROCESSED, CANNED, SHELF STABLE FOODS

5.11 Discussion

The microbiological examination of canned food products requires a thorough understanding of the sciences of microbiology and food science. The examination itself requires strict aseptic techniques.

There are many books, and scientific articles dealing with the microbiology of canned foods. Individuals performing this analysis should thoroughly review the available literature. (5.3)

Some basic concepts and terminology associated with canned food examination are as follows:

5.111 Commercial Sterility. Canned foods are processed at times and temperatures that allow the finished product to be marketed and stored without microbial spoilage. This does not mean that all of the original bacteria have been destroyed, but rather that all of those capable of growth in the product after processing have been destroyed. For example, thermophilic spores may remain viable, when no mesophilic spores or vegetative cells remain. Such a product will remain for years with no evidence of spoilage, unless it is stored at temperatures of 115-130° F., whereupon swelling or flat sour spoilage may occur because of the growth of the thermophilic bacteria. Many low acid canned meats and meat products contain low numbers of thermophilic spores. Thus, "commercial sterility" is the absence of viable spores or vegetative cells capable of growth in a specific canned product under normal storage conditions. Do not incubate canned foods routinely at 55° C. because the results are usually confusing and provide no sound information. Lots destined for tropical countries are exceptions to this rule.

5.112 Adventitious Contamination. Adventitious contamination may be defined as the contamination of the contents of a can from the environment of the laboratory during analysis. This occurs frequently if the microbiologist has not sterilized the can lid or opening device adequately, or if he is careless while he manipulates his equipment or cultures.

5.113 Product abnormalities. Analyze both normal and abnormal cans, and correlate cultural results with can abnormalities, such as swells, leakers defective seams; and with product abnormalities, as determined by tests for pH, odor, color, gross appearance, and direct microscopic examination.

5.114 Hydrogen swells. Food acids act on the metal of the can to form hydrogen, and in so doing dissolve metal, to give a metallic odor and taste to the product. Usually there is no other abnormality; the product

is not hazardous to health. The fault is common among acid foods, but is not necessarily limited to them, and typically occurs only after prolonged storage.

5.115 Sterile Microbiological Swells. Occasionally, swells containing visibly decomposed product will yield repeated negative cultures even when both organoleptic and microscopic examinations indicate that bacterial decomposition has occurred. High temperatures of storage and increased acidity accelerate bacterial autolysis and death. To keep this possibility to a minimum, analyse swells as promptly as possible.

5.116 Curing Salts. Many canned meats contain curing salts. These consist generally of sodium chloride, sodium nitrite and sodium nitrate. When combined with a meat product and heated, sodium nitrite acts as a preservative against the outgrowth of bacterial spores, particulary clostridial spores. Depressing the pH and increasing the salt concentration enhance the preservative action of sodium nitrite. Many cured meat products are not fully retorted but are rendered "commercially sterile" by a combination of heat, salt, pH and sodium nitrite. Spoilage in canned cured meats is rare. When it occurs, it is usually the result of incomplete curing rather than inadequate heating. The heat processes employed for cured, canned, shelf stable meats are unique in that they are not always designed to destroy mesophilic bacterial spores, but merely to inhibit their outgrowth.

5.117 Processing and Handling Faults. Not all can abnormalities are caused by chemical or microbial processes. Hermetically sealed cans and their contents generally respond to physical changes and the laws of hydraulics, pressures and temperature. Some of these problems are:

5.1171 Overfilling: With little or no head space for expansion of contents, overfilled cans may appear abnormal at room temperatures. They tend to swell more upon slight increases in temperature.

5.1172 Closure at low temperatures: Product closed at low temperature has little or no vacuum. Packs of this nature may show flippers and springers immediately after processing.

5.1173 Altitude: Low vacuum cans shipped from areas of high atmospheric pressures to areas of lower atmospheric pressures may appear slightly swollen.

5.1174 Changes in Storage Temperatures: Storage of cans, particularly low vacuum cans, at elevated temperatures causes product expansion and vacuum reduction. Thus, in the laboratory, cool incubated cans to room temperature before classifying them according to 5.12.

5.1175 Can damage: Denting may distend the end panel. Describe dented cans in the report.

5.12 Classification of Rigid Metal Containers.

Keep cans at normal room temperature for initial classification. Classify into 5 categories in accordance with the following descriptions:

5.121 Normal. Both ends are slightly concave and show no tendency to become convex.

5.122 Flippers. Both ends are slightly concave. However, when struck sharply on a flat surface, the end struck bulges slightly, but when pressed with the thumb, the end returns to its original concave condition.

5.123 Springers. One end is convex. Pressing the convex end forces the opposite (normal) end to the convex condition. The end on which pressure has been exerted remains flat upon removal of the thumb.

5.124 Soft Swells. Both ends are convex. Ends may be pressed inward by medium hard thumb pressure but spring back to original position upon removal of thumb.

5.125 Hard Swells. Both ends are convex. Ends are rigid and do not respond to medium hard thumb pressure.

5.13 Classification of Glass Containers.

Classify glass containers by the condition of the lid only. Do not strike such containers against a surface as directed under flipper classification. (5.112). Instead, shake the container abruptly to cause the contents to exert force against the lid; this procedure occasionally reveals a flipper. Examine the contents through the glass prior to opening the container.

5.14 Incubation of Containers.

Incubate normal cans at 35° C. for 10 days. If abnormal cans have been reported in a pack or lot, this incubation may reproduce the abnormality and thereby document progressive microbiological processes. Examine the incubated cans daily. Remove any swells from the incubator as they develop and culture them. After the incubation period, cool the cans to room temperature and reclassify. If no change occurred during the incubation period, terminate the analysis of the incubated cans.

5.15 Examination of Containers (Cans and Glass).

5.151 Cans. Before opening and examining the contents of a can, examine the seams. Using a felt marker, make three slash marks at irregular intervals across the label and the code end seam. Correlate any residues on the label with suspicious areas on the side panel by returning the label to its exact position relative to the slash marks.

Examine the code end, non-code end and side panel seams for any evidence of flaws or physical damage. Examine all non-seam areas of the can for any evidence of physical damage. Examine the embossed code for any evidence of puncturing. Mark and record suspect areas.

5.152 Glass. Prior to opening the jars, strip the label and examine the container grossly with a good light source. Test the lid very gently to determine its security. Record any surface residues and their locations. Microorganisms may enter small cracks in the glass caused by impact or freezing of contents. Microorganisms may have entered the area of the lid. After examination as in Section 5.18, examine the lid and the glass rim of the jar. Look for flaws in the sealing ring or compound inside the lid; for food particles lodged between the glass and the lid; for chips or uneven areas in the glass rim.

5.16 Glossary of Seam Terminology and Defects.

Base Plate. Part of a closing machine which supports cans during seaming operation.

Beaded Can. A can which is re-enforced by having ring indentations around the body. The bead tends to keep the can cylindrical and helps to eliminate paneling of the can body.

Body. Principal part of a container - usually the largest part in one piece containing the sides. May be round, or cylindrical, or other shape.

Body Hook. Can body portion of double seam. Prior to seaming this portion was the flange of the can.

Bottom Seam. Also known as the factory end seam. The double seam of the can end put on by the can manufacturer.

Can Size. Two systems are commonly used to denote can size:

An Arbitrary system (1, 2, etc.) with no relation to finished dimension.

A system indicating the nominal finished dimensions of a can (307 x 409). In this, the first group of numbers (307) indicates diameter and the second group (409) the can height.

The first number on the left in each case is inches, and the next two numbers are in 1/16 of an inch. Hence, the can has a diameter of 3-7/16 and a height of 4-9/16.

Chuck. Part of a closing machine which fits inside the countersink and in the chuck ring of a can lid or end during the seaming operation.

Closing Machine. Also known as double seamer. Machine which double seams the can end onto the can bodies.

5.16 (con. #1)

Cocked Base Plate. A base plate on the seamer which is not parallel to the chuck. This results in a top double seam having a body hook uneven in length.

Cocked Body. A can body which is not a perfect cylinder, i.e., the open ends of the cylinder are not at right angles to the body. The defect results in body hooks of uneven length at both ends. When the body is long on one end, it will be short on the other end.

Compound. Rubber or other material applied inside the end curl to aid in forming an hermetic seal when the end is double seamed on the can body.

Countersink. On a seamed end, the perpendicular distance from the outermost end panel to the top of the seam.

Cover. Can end placed on can by packer. Also known as top, lid, packer's end, canner's end.

Cover Hook. That part of double seam formed from the curl of the can end.

Cross Over. The portion of a double seam at the lap.

Cross Section. Referring to a double seam, a section through the double seam.

Curl. The semi-circular edge of a finished end prior to double seaming. The curl forms the cover hook of the double seam.

Cut Code. A break in the metal of a can end due to improper embossing-marker equipment.

Cut-over. Sharp bend or break in the metal at the tip of the countersink. The cut-over occurs during seaming due to excess metal being forced over the top of the seaming chuck. Usually caused by heavy laps; i.e., laps containing excessive solder, but may be due to improper seamer adjustments.

Dead-Head. An incompletely rolled finished seam. Also known as skip, skid, spinner.

Double Seam. The joint between the end and the can body formed by rolling the curl under the flange (1st operation) and then pressing the metal together (2nd operation).

Droop. A smooth projection of double seam below the bottom of a normal seam. While droops may occur at any point of the seam, they usually are evident at the side seam lap. A slight droop at the lap may be considered normal because of additional plate thicknesses incorporated into the seam structure.

5.16 (Con. #2)

Factory End. Bottom or can manufacturer's end.

False Seam. A small seam breakdown where the cover hook and body hook are not over-lapped; i.e., no hooking of body and cover hooks. See Knockdown Flange for complete distinguishing characteristics.

Feather. Beginnings of a cut-over. At the top of the container's countersink, the metal is forced over the seaming chuck, forming a sharp edge that may be detected with the fingernail. Commonly referred to as "Sharp Edge."

First Operation. The first operation in double seaming. In this operation, the curl of the end is tucked under the flange of the can body which is bent down to form cover hook and body hook, respectively.

Flange. The portion of the can body which is flared at each end to facilitate double seaming.

Fluted. A condition of a can body wherein a many sided polygon, instead of a smooth circumferential circle, is obtained as a cross section of the body.

Headspace. The free space above the contents of a can.

a. Gross Headspace. Space above the contents from the top of the double seam.

b. Net Headspace. Gross headspace less 3/16 inch. This approximates the space above the contents below the can lid.

Heavy Lap. A lap containing excess solder. Also called a thick lap.

Hooks.

1. The bent over edges of a body blank, which form the side seam lock.

2. The body and cover hooks in a double seam.

Internal Enamel. A coating applied to the inside of the can to protect the can from action by the contents or to prevent discoloration. A lacquer is usually clear; an enamel is pigmented and opaque.

Knowndown Flange. A seam defect in which the flange is bent against the body of the can. Thus, the cover hook is not tucked inside the body hook, but lies outside of it. False seams, knockdown flanges and soft crabs are degrees of the same effect. In order to distinguish the degree of the defect, the following terminology is suggested:

5.16 (Con. #3)

False Seam. The cover hook and body hook are not tucked for a distance of less than an inch. Thus, it may not be possible to detect a false seam until the can is torn down.

Knockdown Flange. As above, but more than an inch in length. Body hook and cover hook in contact, but not tucked.

Soft Crab. A defect in which the body of the can is broken down and does not contact the double seam. Thus, there is a wide open hole in the can below the double seam where the body was not incorporated into the seam.

Knockout Mark. A dent in the center of the can end resulting from a blow by the knockout rod. Such a mark indicates that the cans are sticking to the chuck and may be caused by a cut-over.

Knockout Rod. Rod extending through the chuck of a double seamer responsible for forcing the can end on the body before seaming and removing the can from the chuck at completion of the operation.

Lap. The soldered but not locked portions of a side seam at the ends of the can body, to allow for easier seaming. The lap on one edge of the blank is notched to further ease the double seaming operation.

Lid. See Cover.

Lip, Spurs or Vees. Irregularities in the double seam due to insufficient or sometimes absence of overlap of the cover hook with the body hook, usually in small areas of the seam. The cover hook metal protrudes below the seam at the bottom of the cover hook in one or more "V" shapes.

Mislock. A poor or partial side seam lock, due to improper forming of the side seam hooks.

Notch. The cut-away portions on the outside corners of the laps on one side of the body blank to reduce the number of layers of plate brought together in seaming.

Oozer. An imperfect can which allows the escape of the contents through the seam.

Open Lap. A lap which failed due to various strains set up during manufacturing operations. Also caused by improper cooling of the solder (See Weak Lap). A lap which is not properly soldered so the two halves are not properly joined.

Over Lap. The distance the cover hook laps over the body hook.

5.16 (Con. #4)

Panel. A flattening of the side of a can due to abuse, weak plate, excessive internal vacuum, external pressure, or a combination of these. Also used to define concentric (expansion) rings in can ends.

Peaking. Permanent deformation of the expansion rings on the ends of the cans due to rapid reduction of steam pressure at the conclusion of processing. Such cans have no positive internal pressure and the ends can be forced back more or less to their normal position.

Perforation. Holes in the metal of a can resulting from the action of acid in food on metal. Perforation may come from inside due to product in the can, or from outside due to material spilled on the cans.

Pleat. A fold in the cover hook metal which extends from the edge downward toward the bottom of the cover hook and sometimes results in a sharp droop, vee, or spur.

Pressure Ridge. A ridge formed on the inside of the can body directly opposite the double seam, as a result of the pressure applied by the seaming rolls during seam formation.

Pucker. A condition which is intermediate between a wrinkle and a pleat in which the cover hook is locally distorted downward without actual folding. Puckers may be graded the same way as wrinkles.

Sanitary Can. Can with one end attached, the other end put on by the packer after the can is filled. Also known as packer's can or open top can.

Seam Width. The maximum dimensions of a seam measured parallel to folds of the seam. Also referred to as the seam length or height.

Seam Thickness. The maximum dimension measured across or perpendicular to the layers of the seam.

Second Operation. The finishing operation in double seaming. The hooks formed in the first operation are rolled tight against each other in the second operation.

Side Seam. The seam joining the two edges of a blank to form a body.

Slipper, Spinner. See Deadhead.

Top Seam. Synonym for packer's end seam.

Uneven Hook. A body or cover hook which is not uniform in length.

Vee. See Lip.

5.16 (Con. #5)

Weak Lap. The lap is soldered and both parts are together. However, strain on this lap, as twisting with the fingers, will cause the solder bond to break.

Worm Holes. Voids in solder usually at the end of the side seam. May extend completely through the width of the side seam.

Wrinkle. The small ripples in the cover hook of a can. A measure of tightness of a seam.

5.17 Glossary of Glass Container Parts. From a manufacturing standpoint, there are three basic parts to a glass container based on the three parts of glass container molds in which they are made. These are the finish, the body, and the bottom.

Finish. The finish is that part of the jar that holds the cap or closure. It is the glass surrounding the opening in the container. In the manufacturing process, it is made in the neck ring or the finish ring. It is so named since in early hand glass manufacturing, it was the last part of the glass container to be fabricated, hence "the finish."

Body. The body of the container is that portion which is made in the "body-mold" in manufacturing. It is the largest part of the container and lies between the finish and the bottom.

Bottom. The bottom of the container is made in the "bottom plate" part of the glass container mold.

The finish of glass containers has several specific areas as follows:

Sealing Surface. That portion of the finish which makes contact with the sealing gasket or liner. The sealing surface may be on the top of the finish, or may be a combination of both top and side seal.

Glass Lug. One of several horizontal tapering protruding ridges of glass around the periphery of the finish that permit specially designed edges or lugs on the closure to slide between these protrusions and fasten the closure securely with a partial turn. The number of lugs will vary with the number of lugs on the closure and their precise configuration is established by the closure manufacturer.

Continuous Thread. A continuous spiral projecting glass ridge on the finish of a container intended to mesh with the thread of a screw-type closure.

5.17 (Con. #1)

Transfer Bead. A continuous horizontal ridge of glass near the bottom of the finish used in transferring the container from one part of the manufacturing operation to another. NOTE: Not all glass containers have transfer beads. Some achieve the transfer in manufacturing through different means.

Vertical Neck Ring Seam. A mark on the glass finish resulting from the joint of matching the two parts of the neck ring. NOTE: Some finishes are made in a one-piece ring and do not have this seam.

Neck Ring Parting Line. A horizontal mark on the glass surface at the bottom of the neck ring or finish ring resulting from the matching of the neck ring parts with the body mold parts.

The characteristic parts of the body of a glass container are as follows:

Shoulder. That portion of a glass container in which the maximum cross-section or body area decreases to join the neck or finish area. Most glass containers for processed foods have very little neck. The neck would be any straight area between the shoulder and the bottom of the bead or, with beadless finishes, the neck ring parting line.

Heel. The heel is the curved portion between the bottom and the beginning of the straight area of the side wall.

Side Wall. The remainder of the body area between the shoulder and the heel.

Mold Seam. A vertical mark on the glass surface in the body area resulting from matching the two parts of the body mold.

The designated parts of the bottom area are normally:

Bottom Plate Parting Line. A horizontal mark on the glass surface resulting from the matching of the body mold parts with the bottom plate.

Bearing Surface. That portion of the container on which it rests. The bearing surface may have a special configuration known as the "stacking feature" which is designed to provide some interlocking of the bottom of the jar with the closure of another jar on which it might be stacked for display purposes.

5.18 Culturing Procedures

5.181 Preparation

5.1811 The analyst. Cover your hair completely with a clean disposable operating room cap. If you have a beard, mustache, or sideburns below the earlobe, cover your facial hair with a mask. Wear a clean laboratory coat and sanitize hands thoroughly prior to opening cans.

5.1812 Opening Devices. Either grind a star chisel (1/4 to 1/2 x 12 to 15 inches) to a point on one end, and fit it with a hardwood handle; or purchase the adjustable Bacti-disc cutter from Wilkens-Anderson Company, 4525 W. Division Street, Chicago, Illinois. A similar device is available from American Can Company, North Genesee Street, Geneva, New York. (Order Number WT2437). Adjust the opening device to provide a hole large enough for entry and transfer of contents, but no larger. Flame the opening device thoroughly, including crevices and shanks that could contact product. Alcohol flaming is not sufficiently hot. After sterilization, carefully protect the device against recontamination.

5.1813 Preparation of Cans Prior to Opening. Select a draft-free area and sanitize the bench surfaces with 3% saponated cresol solution or equivalent. Do not open canned foods in a dusty area. Refrigerate swells prior to opening and wear safety glasses throughout the analysis. Scrub the non-code ends of the can to be opened with scouring powder and a clean paper tissue. This removes bacteria-laden oil and protein residues. Rinse can under tap and shake off excess water.

5.1814 Media. Modified Cooked Meat Medium (20.9) or PE-2 Medium (20.48) Brom Cresol Purple Broth (20.10)

5.182 Analysis.

5.1821 Opening Containers.

5.18211 Normal Cans. Heat the area to be entered with a laboratory burner or propane torch. Shake can to dissipate end panel heat and to distribute contents. Using the pre-sterilized opening device, prepare the desired size entry hole. Transfer contents immediately to the selected media.

Open glass containers having a metal top by the same method.

Caution: The contents from overfilled cans may flow out of the hole onto the surrounding lid surface at the time of opening with the possibility of draining back into the can upon removal of the opening device. Terminate the analysis if this occurs.

5.18212 Swells. Do not flame swells; they may burst. Place the washed container into a large shallow pan with the non-code end up, and the side seam facing away from the analyst. Place a clean tissue over the non-code end and saturate with chlorine solution. (Commercial bleach, containing

approximately 5% available chlorine, diluted 1: 100 with 0.5 M phosphate buffer at pH 6.2). Allow to stand for 20 minutes, remove tissue and shake the can to remove the excess solution and distribute the contents. Using the pre-sterilized opening device, make a small hole in the center of the dome and maintain pressure over the hole. Release the instrument slowly to allow the gas to escape. After the gas pressure has been released, enlarge the opening to the desired size. Transfer contents immediately to the selected media.

5.1822 Transfer of Contents To Media. Transfer contents of the can to appropriate media. Transfer fluids with large orifice pipettes. Transfer 0.2 to 0.5 ml. to each tube of medium. When inoculating tubes designated to grow the anaerobic bacteria, make all inoculations deep. For solid meats or other products that cannot be transferred with pipettes, use suitable sterilized forceps, spatulas, knives, etc. For solid meats, use individual cork borers fitted with cotton tipped applicator sticks to obtain an excellent representative sample. Use the stick as a plunger to express the core into the tubes of media. Sterilize all instruments used in sampling and take care to prevent contamination while opening wrappings and manipulating devices. Transfer a 10-20 gram portion to a sterile tube or jar for reserve. Carry out transferring procedures expeditiously to minimize airborne contamination. Finally, transfer a portion of the contents into a jar or beaker for pH, microscopic and organoleptic examinations. Cover the hole of the can with a sterile petri dish half, secure it with tape and freeze.

5.1823 Inoculation and Incubation of Tubes. Inoculate 2 tubes of Modified Cooked Meat Medium (20.9) or 2 tubes of PE-2 Medium (20.48) and 2 tubes of Brom Cresol Purple Broth (20.10). Incubate one tube of each medium at 35° C. and one tube of each at 55° C. Observe tubes at 24 and 48 hours. Subculture questionable tubes, especially when the product under examination contributes turbidity.

5.1824 Identification of Organisms. Using conventional bacteriological procedures, characterize the microbial flora from the contents of the can in general terms such as: mixed cultures or pure cultures; anerobic or aerobic; spore former or non-spore former; mesophile or thermophile; cocci or rods; etc.

5.1825 Supportive Determinations. Examine the contents of each can after subculturing. Determine the pH and make direct microscopic examination (1% aqueous methylene blue or 0.6% methylene blue in 95% ethyl alcohol). Prepare a spore stain if decomposed contents from a swell show few bacterial cells after staining with methylene blue. Examine the product for odor, color, consistency and texture. Do not taste it. Examine the can lining for blackening, detinning and pitting. When seam leakage is suspected, test the clean empty can with a vacuum micro-leak detector. Compare results on normal and abnormal product.

5.1826 Interpretation of Results. Use Table I and Table II to arrive at possible causes of spoilage based upon laboratory results. The tables do not cover multiple spoilage conditions.

5.1827 Normal pH Values of a Few Canned Meats

Kinds of Food	pH
Beef Stew	5.9
Beef Taco Filling	5.8
Chicken Noodle Soup	6.2 - 6.5
Chicken Soup with Rice	6.7 - 7.1
Chicken Broth	6.8
Chorizos	5.2
Corned Beef	6.2
Corned Beef Hash	5.0 - 5.7
Egg Noodles & Chicken	6.5
Ham	6.0 - 6.5
Pork with Natural Juices	6.3
Roast Beef	5.9
Stuffed Cabbage	5.9
Sloppy Joe	4.4
Vienna Sausage	6.2 - 6.5
Weiners, Franks	6.2

5.2 Examination of Heat Processed, Canned, Non-Shelf Stable Meats

5.21 Discussion

Perishable canned hams, luncheon meats and loaves are hermetically sealed, then heat processed to internal temperatures of not lower than 150° F and usually not higher than 160° F. "Perishable, Keep Refrigerated" must appear on the label of these products. Although they are not shelf stable, good commercial processing destroys vegetative bacterial cells. The combined effects of sodium nitrite, salt, refrigeration, and low oxygen tension retard the outgrowth of the few spores that may survive the process.

5.211 Examination of Containers. See Section 5.15.

5.22 Culturing Procedures.

5.221 Preparation of Cans Prior to Opening. Select a draft free area and sanitize the bench surface with 3% saponated cresol solution or equivalent. Do not open canned foods in a dusty area.

Scrub the side panel near the non-coded end of pullman cans and the non-coded end of pear shaped cans with abrasive cleaner and a clean paper tissue. Rinse can under tap. Place a paper tissue over the scrubbed area and wet with chlorine water (5.18212). Let stand for twenty minutes. Remove the tissue and flame the area with a laboratory burner to evaporate the remaining moisture. Do not flame hard swells; they may burst.

5.222 Opening Containers. Using a pre-sterilized opening device (See Section 5.1812) to cut the desired size entry hole, usually about two inches in diameter.

5.223 Preparation and Dilution of Homogenate. Using presterilized triers, large cork borers, scissors, knives or forceps, remove and weigh 50 grams of the sample into a sterile blender jar. This laboratory utilizes a tail piece (laboratory drain pipe) 1 1/2" in diameter, 10" long, chrome finished, flanged at one end and sharpened on the other (available from hardware or plumbing supply stores). The sharpened tail piece removes a large plug for representative sampling. Prepare the homogenate in accordance with Section 3.3. Cover the container opening with sterile aluminum foil several layers thick and secure with tape. Place the opened sample unit in the freezer until the analysis is complete.

5.224 Aerobic Plate Counts. Prepare two sets of duplicate plates per dilution as in section 3.3. Substitute APT agar (20.49) for one of the sets. Incubate the set containing standard methods agar (20.2) at 35° C for 48 hours. Incubate the set containing APT agar at 20° C for 96 hours. Count and record the results as in section 3.4.

5.225 Gas-Forming Anaerobes. Steam tubes of Modified Cooked Meat Medium (MCMM; 20.9) for 10 minutes and cool. From each dilution prepared in 5.224, make a deep, 1 ml. transfer into MCMM tubes using separate pipettes for each dilution. Begin with a 10^{-1} dilution; subsequent dilutions must be sufficiently high to yield a negative end point. Incubate 48 hours at 35° C and read daily. Consider MCMM tubes showing a bright yellow color, producing gas and containing gram positive or gram variable rods as positive. Report as gas forming anaerobes per gram calculated as the reciprocal of the highest positive dilution. If skips occur, disregard the final actual dilution and calculate it at the dilution where the skip occurred.

5.226 Enterococci. From each dilution prepared in 5.224, transfer 1 ml. into a tube of KF broth (20.50) using separate pipettes for each dilution. Begin with a 10^{-1} dilution; subsequent dilutions must be sufficiently high to yield a negative end point. Incubate at 35° C for 48 hours. Tubes showing a yellow color, turbidity and buttoning of growth are presumptive positives. Confirm all presumptive positives microscopically. Either wet mounts examined under low light or gram stained preparations are suitable for microscopic determinations. Microscopic determinations yielding cells with ovoid streptococcal morphology shall be considered confirmed. Report confirmed enterococci as enterococci per gram calculated as the reciprocal of the highest positive confirmed dilution. If skips occur, disregard the final actual dilution and calculate it at the dilution where the skip occurred.

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Table 1

KEY TO PROBABLE CAUSE OF SPOILAGE IN CANNED FOODS

Group 1. Low-Acid Foods -- pH Range 5.0 to 8.0

Condition of Cans	Characteristics of Material in Cans						
	Odor	Appearance	Gas (CO_2 & H_2)	pH	Smear	Cultures	Diagnosis
Swells	Normal to "metallic"	Normal to frothy (Cans usually etched or corroded)	More than 20% H_2	Normal	Negative to occasional organisms	Negative	Hydrogen swells
	Sour	Frothy; possibly ropy brine	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci, yeasts or molds	Growth, aerobically and/or anaerobically at 30° C., and possibly at 50° C.	Leakage
	Sour	Frothy; possibly ropy brine, food particles firm with uncooked appearance	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci and yeasts	Growth, aerobically and/or anaerobically at 30° C., and possibly at 50° C. (If product received high exhaust, only spore formers may be recovered)	No process given
	Normal to sour-cheesy	Frothy	H_2 and CO_2	Slightly to definitely below normal	Rods, med. short to med. long, usually granular; spores seldom seen	Gas, anaerobically at 50° C., and possibly slowly at 30° C.	Post-processing temperature abuse Thermophilic anaerobes
	Cheesy to putrid	Usually frothy with disintegration of solid particles	Mostly CO_2; possibly some H_2	Slightly to definitely below normal	Rods; usually spores present	Gas anaerobically at 30° C.	Underprocessing - mesophilic anaerobes (possibility of *Cl. botulinum*)
	Slightly off - possibly ammoniacal	Normal to frothy		Slightly to definitely below normal	Rods; spores occasionally seen	Growth, aerobically and/or anaerobically with gas at 30° C. and possibly at 50° C. Pellicle in aerobic broth tubes. Spores formed on agar and in pellicle.	Underprocessing - *B. subtilis* type
No vacuum and/or Cans buckled	Normal	Normal	No H_2	Normal to slightly below normal	Negative to moderate number of organisms	Negative	Insufficient vacuum, caused by: 1) Incipient spoilage, 2) Insufficient exhaust, 3) Insufficient blanch, 4) Improper retort cooling procedures, 5) Over fill
Flat cans (0 to normal vacuum	Normal to sour	Normal to cloudy brine		Slightly to definitely below normal	Rods, generally granular in appearance; spores seldom seen	Growth without gas at 50° C. Spore formation on nutrient agar	Post-Processing temperature abuse Thermophilic flat sours.
	Normal to sour	Normal to cloudy brine; possibly moldy		Slightly to definitely below normal	Pure or mixed cultures of rods, coccoids, cocci or mold	Growth, aerobically and/or anaerobically at 30° C., and possibly at 50° C.	Leakage

Table 2
KEY TO PROBABLE CAUSE OF SPOILAGE IN CANNED FOODS

Group 2. Semi-Acid Foods - pH Range 4.6 to 5.0

Condition of Cans	Characteristics of Material in Cans: Odor	Appearance	Gas (CO_2 & H_2)	pH	Smear	Cultures	Diagnosis
Swells	Normal to "metallic"	Normal to frothy. (Cans usually etched or corroded)	More than 20% H_2	Normal	Negative to occasional organisms	Negative	Hydrogen swells
	Sour	Frothy; possibly ropy brine	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci, yeasts or mold	Growth, aerobically and/or anaerobically at 30° C., and possibly at 50° C.	Leakage
Note: Cans are Sometimes flat	Sour	Frothy; possibly ropy brine, food particles firm with uncooked appearance	Mostly CO_2	Below Normal	Pure or mixed cultures of rods, coccoids, cocci or yeasts	Growth, aerobically and/or anaerobically at 30° C., and possibly growth at 50° C. (If product received high exhaust, only spores formers may be recovered)	No process given
	Normal to sour - cheesy	Frothy	H_2 and CO_2	Slightly to definitely below normal	Rods - med short to med. long, usually granular; spores seldom seen	Gas anaerobically at 50° C. and possibly slowly at 30° C.	Post-processing temperature abuse. Thermophilic anaerobes
	Normal to cheesy to putrid	Normal to frothy with disintegration of solid particles	Mostly CO_2; possibly some H_2	Normal to slightly below normal	Rods; possibly spores present	Gas anaerobically at 30° C. Putrid odor	Underprocessing - mesophilic anaerobes (possibility of Cl. botulinum)
	Slightly off - possibly ammoniacal	Normal to frothy		Slightly to definitely below normal	Rods; occasionally spores observed	Growth, aerobically and/or anaerobically with gas at 30° C. and possibly at 50° C. Pellicle in aerobic broth tubes. Spores formed on agar and in pellicle	Underprocessing - B. subtilis type
	Butyric acid	Frothy, large volume gas	H_2 and CO_2	Definitely below normal	Rods - bipolar staining; possibly spores	Gas anaerobically at 30° C. Butyric acid odor	Underprocessing - butyric acid anaerobe
No vacuum and/or Cans buckled	Normal	Normal	No H_2	Normal to slightly below normal	Negative to moderate number of organisms	Negative	Insufficient vacuum, caused by: 1) Incipient spoilage, 2) Insufficient exhaust, 3) Insufficient blanch, 4) Improper retort cooling procedures, 5) Over fill
Flat cans (o to normal vacuum)	Sour to "medicinal"	Normal to cloudy brine		Slightly to definitely below normal	Rods - possibly granular in appearance	Growth without gas at 50° C. and possibly at 30° C. Growth on thermoacidurans agar	Underprocessing B. coagulans
	Normal to sour	Normal to cloudy brine; possibly moldy		Slightly to definitely below normal	Pure or mixed cultures of rods, coccoids, cocci or mold	Growth, aerobically and/or anaerobically at 30° C., and possibly at 50° C.	Leakage

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6. DETERMINATION OF ANTIBIOTIC RESIDUES IN ANIMAL TISSUES

The following procedure is a modified version of that developed by the Food and Drug Administration: "Information for Assay and Reporting of Data Pertaining to Antibiotic Residues in Milk, Dairy Products, and Animal Tissue."
Revised. FDA. October, 1968.

6.1 EQUIPMENT AND MATERIALS

6.12 Test Organisms

(1) Penicillin susceptible----*Sarcina lutea* ATCC 9341a
(2) Tetracycline susceptible--*Bacillus cereus* var.- *mycoides* ATCC 11778
(3) Streptomycin susceptible--*Bacillus subtilis* ATCC 6633
(4) Tetracycline resistant---*Staphylococcus epidermidis* ATCC 12228
(5) Streptomycin resistant----*Sarcina lutea* ATCC 9341a
(6) Neomycin susceptible------*Staphylococcus epidermidis* ATCC 12228
(7) Erythromycin susceptible--*Sarcina lutea* ATCC 9341a
(8) Neomycin resistant--------*Sarcina lutea* ATCC 9341
(9) Erythromycin resistant---*Sarcina lutea* ATCC 15957

6.13 Laboratory Equipment

(1) Stainless steel (SS) bio-assay plates with 6 wells spaced at 60° intervals.*
(2) Plastic Petri dishes, 25 x 100 mm.
(3) Antibiotic media. (Section 20)
(4) Bausch & Lomb Spectronic 20 with round cells 1.2 cm inside diameter or comparable spectrophotometer or colorimeter.
(5) Blenders or grinders, comparable to Hobart Kitchen Institutional type with 25 or more interchangeable heads.
(6) Roux bottles.
(7) Zone reading device.

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* Item No. D-3299; Scientific Glass Apparatus, 735 Broad Street, Bloomfield, New Jersey.

6.13 (Con.)

(8) Sterile glass beads.
(9) Forceps for SS bio-assay plates.
(10) Wooden trays, base 12 1/2 x 5 1/8 inches of perforated hard board. Frame consists of 2 pieces 3/4 x 3/4 x 3 1/2 inches and 2 pieces 3/4 x 3/4 x 12 1/2 inches. Inside dimensions are 11 x 3 1/2 x 3/4 inches.
(11) Weighing flasks.
(12) Desiccator.
(13) Semilogarithmic graph paper, 2 cycle, 11 x 16 1/2 inches.
(14) Pasteur pipettes.
(15) Rubber bulbs, small, for use with Pasteur pipettes.
(16) Flasks, volumetric, 25, 50, and 100 ml.
(17) Flasks, Erlenmeyer, 250/300 ml.
(18) Tubes, glass, 25 x 175 mm, disposable.
(19) Pipettes, graduated, assorted sizes.

6.14 Reagents

(1) Antibiotic Standards.*
(2) Antibiotic free tissue, same species as sample.
(3) 1% phosphate buffer, pH 6.0 (± 0.1). Dissolve 8.0 gm. monobasic potassium phosphate and 2.0 gm. dibasic potassium phosphate in distilled water and dilute to 1 liter with distilled water.
(4) 0.1M phosphate buffer, pH 8.0 (± 0.1). Dissolve 16.73 gm. dibasic potassium phosphate and 0.523 gm. of monobasic potassium phosphate in distilled water and dilute to 1 liter with distilled water.
(5) 0.1M phosphate buffer, pH 4.5 (± 0.1). Dissolve 13.6 gm. monobasic potassium phosphate in distilled water and dilute to 1 liter with distilled water.
(6) 10% phosphate buffer, pH 6.0 (± 0.1). Dissolve 80 gm. monobasic potassium phosphate and 20 gm. dibasic potassium phosphate in distilled water and dilute to 1 liter with distilled water.
(7) 0.2M phosphate buffer, pH 8.0 (± 0.1). Dissolve 33.46 gm. dibasic potassium phosphate and 1.046 gm. of monobasic potassium phosphate in distilled water and dilute to 1 liter with distilled water.

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* Obtain authentic antibiotic reference standards from United States Pharmacopeia, 4630 Montgomery Avenue, Bethesda, MD 20014, and the Committee on National Formulary, American Pharmaceutical Association, 2215 Constitution Avenue, NW., Washington, D.C. 20037.

(8) 0.01 N HCl
(9) 0.1 N HCl
(10) 1.0 N NaOH
(11) 0.85% NaCl solution. (Sterile saline)
(12) Penicillinase
(13) Tissue buffers. Made with one part appropriate tissue and four parts appropriate buffer. (For controls, tissue must be antibiotic free).

6.2 General Instructions

All glassware and equipment must be chemically clean. First, test tissue controls used to prepare the standard curve or to dilute the unknowns to ensure that they exhibit no activity on the appropriate assay plates. The control should be the same type of tissue being tested. To standardize the microbial suspensions, use an instrument such as a Bausch & Lomb Spectronic 20 with a round cell 1.2 cm., inside diameter or a Beckman Model B Spectrophotometer with a 1.0 cm. square cell, both set at a wave length of 580 mmu. Use the light transmission readings and the amount of inoculum recommended as guides only. Prepare test plates with varying concentrations of the standardized suspension to determine the amount of inoculum that allows the most desirable zones of inhibition as described for each assay.

6.21 Working Standards

Follow the label directions for preparation and storage of the standards. Prepare stock solutions by weighing carefully, in an atmosphere of 50% relative humidity or less, a small amount of the standard and dilute the weighted powder in the appropriate diluent to obtain a solution of a convenient concentration.

6.22 Standard Curve

Prepare a standard curve with antibiotic-free tissue, comparable to that under test. Dilute the control tissue described for the particular method and test this dilution on the appropriate plates to ensure that no zones of inhibition occur. Prepare the standard curve simultaneously with the assay solution. Prepare concentrations of the stock solution (described for each antibiotic). Use the indicated concentration as the reference concentration. Prepare plates with the appropriate base agar layer and the appropriate seed agar layer, (described for each antibiotic). Allow the media to harden on a flat, level surface. Place bio-assay plates on each Petri dish. Fill three alternate wells with the reference concentration and the other three wells with one of the other concentrations of the standard. Use three plates for each concentration required

for the standard curve, except for the reference standard, for a total of 12 plates. Incubate the plates overnight at the appropriate temperature and read the diameters of the inhibition zones. For each set of three plates, average the nine readings of the reference concentration and the nine readings of the concentration being tested. The average of all 36 readings of the reference concentration from the 12 plates is the correction point for the curve.

Correct the average value obtained for each concentration to the appropriate figure if the reference concentration reading on that set of three plates is the same as the correction point. Thus, if in correcting the second concentration of the standard curve, the average of the 36 readings of the reference concentration is 20.0 mm and the average of the nine readings of the reference concentration of this set of three plates is 19.8 mm, the correction is +0.2 mm. If the average of the second concentration on the same three plates is 17.0 mm, the corrected value is 17.2 mm. Plot the corrected values, including the correction point, on semilogarithric graph paper, using the log scale for the concentration and the arithmetic scale for the zone diameters. Draw a line of best fit by inspection or by the following equations:

$$L = (3\,a + 2\,b + c - e\,) / 5$$
$$H = (3\,e + 2\,d + c - a\,) / 5,$$

where L and H equal calculated zone diameters for the low and high concentrations, respectively, on the standard response line; a, b, c, d and e equal the corrected average zone diameters for each concentration on the response line, where a equals the lowest concentration of antibiotic used; and e equals the highest concentration used.

6.23 Determination and Calculation of Potency

To calculate the antibiotic content of a sample, average the zone readings of the standard and the zone readings of the sample on the three plates. If the sample gives a larger average zone size than the average of the standard, add the difference between them to the zone size of the reference standard on the curve. If the average sample value is lower than the standard value, subtract the difference between them from the zone size of the reference standard on the curve. From the curve, read the concentration corresponding to this adjusted sample zone size. Take dilutions into consideration in calculating the final potency of the sample. In those instances where the standards are prepared in a manner which differs from that of the sample, conduct recovery experiments.

6.24 Recovery Experiments

Using control samples of the product to be tested, prepare several samples to which have been added varying amounts of the antibiotic. The concentrations chosen should fall within the range of concentrations of the standard curve. Carry out the extraction procedures with these samples in parallel with the unknowns being tested. Determine the mean recovery of the added antibiotic and use this as a correction factor in the calculation of the potency of the antibiotic in the unknown samples. As an example, if the mean recovery is 50%, apply a correction factor of 100/50 in the calculation of potency of an antibiotic in an unknown sample.

6.25 Controls

It is essential that any antibiotic activity detected derives from the sample and not from the environment. Always include controls to indicate the degree of precision and accuracy of the determinations to be reported. The lowest concentration recommended in the series of standard concentration described for each assay procedure is intended to be a control which may not be detectable. The next higher concentration should be positive and indicate the test sensitivity. Tissues may have inhibitory effects of themselves and, if so, the sensitivity of the method is that amount of antibiotic which produces a significantly greater response than does the tissue itself.

6.26 Preparation of Samples

Preferably, blend 10 grams of sample tissue with 40 ml. of appropriate buffer for one minute. Due to natural inhibition of some tissues, it may be necessary to use a higher dilution. Allow the blend to extract for a minimum of 45 minutes. Decant and filter the clear portion of the extract.

Or, alternately, grind the sample tissue, weigh and dispense 10 gm of the tissue into glass tubes, 25 x 175 mm. (6.13), add 40 ml. of appropriate buffer. Continue as with the blender, except that filtration is not necessary unless the extract is not clear.

6.3 INHIBITION PROCEDURES

6.311 Penicillin Assay Procedure

6.3111 Preparation of Bacterial Suspension

Maintain the test organism, Sarcina lutea (ATCC 9341a), as a stock culture on agar slants of Medium No. 1 (20.11) and transfer to a fresh slant approximately once every two weeks. Prepare a suspension as follow:

6.3118 Calculation of Potency

Proceed as described in Sections (6.23 and 6.24).

6.312 Tetracyclines Assay Procedure (See Section 6.6 for TLC determination and separation of Tetracyclines).

6.3121 Preparation of Bacterial Spore Suspension

The test organism is Bacillus cereus var. mycoides, ATCC 11778, grown at 30° C. and maintained in the refrigerator on antibiotic medium #8 (20.13). Transfer growth from a freshly grown slant with 2-3 ml. of distilled water to a Roux bottle containing 300 ml. of medium #8, and incubate at 35° C. for 18-24 hours and then at room temperature for the remainder of one week. Harvest the growth with approximately 25 ml. sterile distilled water and heat for 30 minutes at 70° C. Wash the culture three times with sterile distilled water by centrifuging and decanting. Heat the residual spores for 30 minutes at 65° C. and resuspend in 30 ml. sterile distilled water. Keep this stock solution refrigerated. Determine by trial plates the per cent of spore suspension necessary to give optimum sensitivity (usually 0.03-0.1%). The spore suspension may be used indefinitely if protected from evaporation and contamination.

6.3122 Preparation of Plates

Add 10 ml. of medium #8 to each petri dish, distribute evenly and allow to harden. Add 4.0 ml. of medium #8 which has been seeded with a suspension of B. cereus var. mycoides and incubated in a water bath at 49-50° C. for 45 minutes. Fill the well for a standard curve and sample determination. Use pH 4.5 phosphate buffer (6.14 (5)) for sample preparation. Incubate at 30° C. (± 1°).

6.3123 Stock Solution

Dissolve approximately 40 mg. of accurately weighed standard in sufficient 0.01 N HCl to give a stock solution of 1000 mcg per ml. The equation used in determining the volume of buffer to add to weighed standard of penicillin may be used to determine the amount of 0.01 N HCl to be added (6.3114). Store the stock solution under refrigeration no longer than seven days.

6.3124 Standard Curve

Dilute the stock solution with enough pH 4.5 buffer to obtain concentrations of 0.08, 0.16, 0.32, 0.64 and 1.28 mcg/ml. The reference concentration is 0.32 mcg/ml. The following dilution method is suggested:

From (mcg/ml)	Take (ml)	Dilute in Vol. flask (ml)	resulting conc. (mcg/ml.)
1000	5.0	50	100.0
100	2.0	50	4.00
4.0	8.0	25	1.28
4.0	4.0	25	0.64
4.0	2.0	25	0.32
4.0	1.0	25	0.16
4.0	1.0	50	0.08

6.3125 Preparation of Sample

Proceed as described in Section (6.26), using the appropriate amount of buffer indicated in Table I, "Details of the Assay Methods."

6.3126 Calculation of Potency

Proceed as described in Sections (6.23 and 6.24).

6.3127 Tetracyclines Resistant Indicator Organism

The same organism is utilized for tetracycline, chlortetracycline and oxytetracycline.

(1) Preparation of Bacterial Suspension. The test organism is Staphylococcus epidermidis, ATCC 12228. Maintain, grow and harvest the organism in the same manner described for Sarcina lutea, ATCC 9341a, except grow on medium #8 (20.13) in the Roux bottles. (6.3111)

(2) Preparation of Plates. Using medium #8 for both base and seed layer, prepare the plates in the same manner as described in 6.26, using the optimum amount of bacterial suspension (usually 1-2 ml.) to be added to 100 ml. of agar. Do not incubate culture in water bath as done with B. cereus. Incubate at 30° C. (± 1°).

6.313 Chlortetracycline

Proceed as for tetracycline (6.3127), except use chlortetracycline as the standard.

6.314 Oxytetracycline

Proceed as for tetracycline (6.3127), except use oxytetracycline as the standard.

6.315 Streptomycin Assay Procedures

6.3151 Preparation of Bacterial Suspensions.

The test organism is Bacillus subtilis, ATCC 6633. Maintain and grow the organisms at 37° C. in the same manner as described for B. cereus var. mycoides (6.3121), using medium #1 with 300 mg. of $MnSO_4H_2O$ per liter of media. Harvest from the Roux bottle and wash three times with sterile saline by centrifuging and decanting. Reconstitute the sediment with 30 ml. of sterile saline and heat shock the spore suspension by heating for 30 minutes at 70° C. Maintain the suspension under refrigeration. Determine optimum sensitivity as with B. cereus (6.312).

6.3152 Preparation of Plates

Using medium #5 (20.14) for base and seed layer, prepare the plates as described in 6.3122, using the optimum amount of bacterial suspension to be added to 100 ml. of agar, and incubate in a water bath at 49-50° C. for 75 minutes. To analyze for streptomycin and/or penicillin, add penicillinase to the seed layer. Incubate at 30° C. (± 1°).

6.3153 Standard Curve

Dry 30-40 mg. of streptomycin sulfate standard for three hours at 60° C. in a vacuum oven at a pressure of 5 mm. of mercury or less. Determine the accurate dry weight and dissolve in enough distilled water to give a stock solution of 1,000 mcg/ml. Calculate the amount of water to be added by the formula used in Section 6.22. Keep the stock solution under refrigeration no longer than 30 days. Dilute the stock solution in pH 8.0 buffer 6.14 to give concentrations of 0.125, 0.25, 0.50, 1.0, 2.0, and 4.0 mcg/ml. Use only the last five concentrations in plotting the response line on semilog paper. Use the 0.125 mcg/ml. to determine the low level of detection. The reference concentration is 1.0 mcg/ml. Use the following dilution method:

From (mcg/ml)	Take (ml)	Dilute in Vol. flask	Resulting conc. (mcg/ml)
1000	2.5	100	25.0
25	4.0	25	4.0
25	2.0	25	2.0
25	1.0	25	1.0
25	1.0	50	0.5
25	0.5	50	0.25
25	0.5	100	0.125

6.3154 Streptomycin Resistant Indicator Organism

The test organism is Sarcina lutea ATCC 9341a. Treat in the same manner as S. lutea in 6.3111, except use medium #5 (20.14) in Roux bottles, base and seed layers.

(1) Preparation of Sample. Proceed as described in Section 6.26, using the appropriate amount of buffer indicated in Table I, "Details of the Assay Methods."

(2) Calculation of Potency. No potency calculation required; use organism as a resistant indicator control.

6.316 Erthromycin Assay Procedure

6.3161 Preparation of Bacterial Suspension

The test organism is Sarcina lutea (ATCC 9341a). Prepare a suspension of the organism in the manner described under Penicillin Assay, Section 6.3111. Before the actual assay, determine by trial plates the optimum amount (usually 0.1 - 0.5 ml.) of the bulk suspension to be added to 100 ml. of medium #11 (20.47) which has been melted and cooled at 49° C. Store the suspension in the refrigerator no longer than two weeks.

6.3162 Preparation of Plates

Add 10 ml. of medium #11 (20.47) to each petri dish, distribute evenly, and allow to harden. Then add 4 ml. of medium #11 which has been seeded with a suspension of Sarcina lutea. Proceed as described for preparation of plates in Section 6.3111. Incubate at 30° C. for 16-18 hours.

6.3163 Standard Curve

Prepare a stock solution by dissolving 30-50 mg. of erythromycin standard in 2 ml. of methanol and then adding sufficient 0.2M pH 8.0 phosphate buffer (6.14(7)), to give a concentration of 1000 mcg/ml. Use the formula in Section 6.22. Dilute the stock solution in 0.2M pH 8.0 buffer to give concentrations of 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 mcg/ml. The reference concentration is 0.2 mcg/ml. Prepare a standard curve as described in Section 6.22, using the above concentrations.

6.3164 Preparation of Sample

Proceed as described in Section 6.26, using the appropriate amount of buffer in Table I, "Details of the Assay Methods."

6.3165 Calculation of Potency

Proceed as described in Sections 6.23 and 6.24.

6.3166 Erythromycin Resistant Indicator Organisms

Treat the test organism (Sarcina lutea ATCC 15957), in the same manner as S. lutea ATCC 9341a in Section 6.3111, except use medium #11 (20.47) in Roux bottles, base and seed layers.

(1) Preparation of Sample. Proceed as described in Section 6.26, using the appropriate amount of buffer indicated in Table I, "Details of the Assay Methods."

(2) Calculation of Potency. S. lutea, ATCC 15957 is resistant to erythromycin. No potency calculation required; use organism as a resistant indicator control.

6.317 Neomycin Assay Procedure

6.3171 Preparation of Bacterial Suspensions

The test organism is Staphylococcus epidermidis (ATCC 12228), maintained as stock solution on agar slants of medium #1 (20.11). Transfer the growth from a fresh slant with 2.0 ml. of sterile physiological saline to a Roux bottle containing 300 ml. of medium #1 and incubate for 18-24 hours at 32-35° C.

Harvest the growth from the surface of the agar with 50 ml. of sterile saline (6.14(11)). Determine the dilution of the suspension to be used (usually 1:25) that will give 25% light transmission. Before the actual assay, determine by trial plates, the optimum amount (usually 1.0-2.0 ml.) of the dilution of the suspension to be added to 100 ml. of medium #11 which has been melted and cooled at 49° C. Store the suspension in the refrigerator no longer than one week.

6.3172 Preparation of Plates

Add 10 ml. of medium #11 (20.47) to each petri dish, distribute evenly and allow to harden. Then add 4 ml. of medium #11 which has been seeded with a suspension of Staphylococcus epidermidis. Proceed as described for preparation of plates under Penicillin (6.3113). Incubate at 32-35° C. for 16-18 hours.

6.3173 Standard Curve

Dissolve the weight of working standard in 0.2M potassium phosphate buffer, pH 8.0 (614), to make a stock solution of 1000 mcg/ml. Store the stock solution not longer than 14 days in the refrigerator. Further dilute the neomycin stock solution in pH 8.0 buffer to give final concentrations of 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 mcg/ml. The 0.1 mcg/ml. concentration is the reference concentration.

6.3174 Preparation of Samples

Proceed as described in Section 6.26, using the appropriate amount of buffer indicated in Table I, "Details of the Assay Methods."

6.3175 Calculation of Potency

Proceed as described in Sections 6.23 and 6.24.

6.4 EXAMPLES OF ANALYSIS

6.41 Penicillin in Swine Muscle

6.411 Example I

6.4111 Assay Method: Sarcina lutea cyclinder plate method:

(1) Media

Base layer: 10 ml. of Medium No. 1
Seed layer: 4 ml. of Medium No. 2

(2) Diluent

One part of control muscle (untreated animal) plus four parts of 1% potassium phosphate buffer, pH 6.0.

(3) Standard

U.S.P. Potassium Penicillin G-20 to 30 mg. dissolved in 1% potassium phosphate buffer, pH 6.0, to give a stock solution of 1000 U/ml. Use solution not to exceed three days.

(4) Test Organism

Sarcina lutea (ATCC 9341a) suspension prepared as directed in Section 6.3111.

6.4111 (Con. #1)

(5) Standard Curve

Standard concentrations of 0.0063, 0.0125, 0.25, 0.05, 0.10, and 0.20 U/ml., were prepared by blending 10 gm. of control tissue plus 39 ml. of 1% potassium phosphate buffer, pH 6.0, plus 1.0 ml. of a solution containing 50 times the desired concentration. The 0.05 U/ml. concentration was used as the reference concentration. This was placed in each of three alternate cylinders, and one of the other standard concentrations in the remaining cylinders. Three plates were incubated at 30° C. for 16-18 hours. After incubation, the zones of inhibition were read and corrected average zone sizes obtained. The curve was plotted on semilog graph paper as described in Section 6.22. The standard curve results obtained were as follows (zone sizes in mm.):

Plate	Set #1 U/ml		Set #2 U/ml		Set #3 U/ml		Set #4 U/ml		Set #5 U/ml	
	0.05	0.0063	0.05	0.0125	0.05	0.025	0.05	0.10	0.05	0.20
1	17.6	Neg.	17.6	12.3	17.6	14.1	17.4	19.9	17.5	22.7
	16.8	Neg.	17.3	12.1	17.4	15.0	17.3	19.8	17.1	22.7
	17.0	Neg.	17.2	10.8	17.5	14.8	17.7	20.0	17.4	23.0
2	17.2	Neg.	18.0	11.7	17.2	13.8	16.8	20.5	17.2	24.1
	17.4	Neg.	17.5	11.9	17.5	14.1	16.3	20.8	17.3	24.3
	17.5	Neg.	17.4	12.0	17.4	14.3	16.7	20.7	17.1	23.8
3	17.7	Neg.	17.3	12.2	17.0	13.7	17.1	20.9	17.0	23.5
	16.9	Neg.	17.8	12.0	16.9	14.2	17.1	20.6	17.1	23.7
	16.7	Neg.	16.8	12.1	16.5	13.6	17.4	20.5	17.5	23.1
Average	17.2	Neg.	17.4	11.9	17.2	14.2	17.1	20.4	17.3	23.4
(Grand average of 45 zone diameters of reference concentration = 17.2)										
Corrected Average				11.7		14.2		20.5		23.3

(6) Calculated End Points:

$$\text{High Point} = \frac{3(23.3) + 2(20.5) + 17.2 - 11.7}{5} = 23.3$$

$$\text{Low Point} = \frac{3(11.7) + 2(14.2) + 17.2 - 23.3}{5} = 11.5$$

6.5 **Table 1. Details of the Assay Methods**

Antibiotics	Sample Dilutions	Diluent	Dilution Factor	Final Concentrations for Standard Curve (a)
Penicillin	1 + 4	buffer 1% pH 6.0	5	0.0063, 0.0125, 0.025, 0.05, 0.10, 0.20, U/ml.
Chlortetracycline	1 + 4	buffer 0.1M, pH 4.5	5	0.005, 0.01, 0.02, 0.04, 0.08, 0.16, mcg/ml.
Tetracycline or Oxytetracycline	1 + 4	buffer 0.1 M pH 4.5	5	0.025, 0.05, 0.1, 0.2, 0.4, 0.8 mcg/ml.
Streptomycin	1 + 4	buffer 0.1M pH 8.0	5	0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mcg/ml.
Neomycin	1 + 4	buffer 0.2M pH 8.0	5	0.125, 0.25, 0.5, 1.0, 2.0, 4.0 mcg/ml.
Erythromycin	1 + 4	methanol and buffer (0.2M) pH 8.0		0.025, 0.05, 0.1, 0.2, 0.4, 0.8 mcg/ml.

(a) The underlined concentration is the reference concentration.

6.4111 (Con. #2)

(7) Sample Preparation

Ten grams of sample muscle were blended with 40 ml. of 1% potassium phosphate buffer, pH 6.0, i.e., a 5-fold dilution. The prepared samples were placed in three alternate cylinders on each of three plates and the 0.05 U/ml. standard was placed in the remaining cylinders. The plates were incubated with those plates described under Standard Curve.

(8) Tissue Control

Ten grams of untreated muscle were blended with 40 ml. of 1% potassium phosphate buffer, 6.0 (pH), as a control of this material used to establish the standard curve.

6.412 Example II

In the previous example we used a standard curve procedure which produced the information necessary to establish the test sensitivity and recovery. In this example, due to the lack of sufficient control tissue, the standard curve was prepared in buffer. It became necessary to conduct experiments to demonstrate test sensitivity and recovery.

Solutions of penicillin in buffer were prepared to contain 0.5, 1.0, and 2.0 units of penicillin per ml., respectively. One ml. of each solution was added to separate 10 gram portions of control muscle. Each sample was blended with 39 ml. of 1% potassium phosphate buffer, pH 6.0, to give final solution of 0.01, 0.02, and 0.04 U/ml. equivalent to 0.5, 0.10, and 0.20 U/gm. of muscle, respectively. On each of three plates, two cylinders were filled with the control solution, two cylinders with the sample solution, and the remaining two cylinders with the standard curve reference concentrations. (Results not shown).

6.6 DETECTION AND IDENTIFICATION OF TETRACYCLINES IN TISSUES

6.61 Apparatus

(1) Chromatographic Chamber.
   a. Cylindrical museum jar for paper.
   b. Square or rectangular jar for TLC.
(2) UV light source, preferably a view box.
(3) Chromatographic paper or TLC.
   a. Whatman #1 filter paper, 8 inch.
   b. Eastman Chromogram sheets--6060 silica gel with fluorescent indicator, 20 x 20 cm.

(4) Various tubes, beakers and related glassware.
(5) Micropipettes 2 & 5 microliter (mcl).
(6) Centrifuge.
(7) Steam Bath.
(8) Air Blower.
(9) Exhaust hood.
(10) Manifold air drying (evaporating) apparatus.

6.62 Reagents

(1) Resolving solvent-Nitromethane-Choloroform-Pyridine (20:10:3 by Vol.)
(2) 0.1M monobasic potassium phosphate (KH_2PO_4)
(3) 2.0% Calcium Chloride ($CaCl_2$)
(4) Acid Acetone (0.1% HCl in acetone.)
(5) pH 3.5 MacIlvaine's buffer (3 parts 0.2M Na_2HPO_4, plus 7 parts 0.1M citric acid)
(6) N-buttanol
(7) Methanolic solution of chlortetracycline (CTC), oxytetracycline (OTC) and tetracycline (TC) solutions to contain 0.5 mg/ml. Use these standards in comparison controls.

(NOTE: PYRIDINE IS TOXIC. USE AN EXHAUST HOOD).

6.63 Preparation of Sample

Dice or grind approximately 25 g. of tissue and place in a 150 ml. beaker. For every gram of tissue, add 1.0 ml. of the 0.1M phosphate solution (6.62(2)). Stir the mixture frequently for 15-20 minutes. Decant into a 25 x 20 mm. tube which has a tight fitting stopper. Heat the solution on a steam bath for three minutes. Cool in a water bath, then add 15 ml. of chloroform and shake the tube vigorously for one minute. If necessary, centrifuge to separate the layers. Pipette 10 ml. of the aqueous layer into another stoppered tube; add three drops of the 2.0% $CaCl_2$ solution, three drops of pyridine and 6 ml. of N-butanol. Shake vigorously for one minute and centrifuge to separate layers. Transfer the clear butanol layer to another tube. Place the tube in a beaker of lukewarm water (60° C. maximum) and evaporate to dryness in a stream of air.

6.64 Chromatographic Procedure

6.641 Paper Chromatography

Prepare a sheet of paper by drawing a line of origin parallel to and one inch from, the bottom edge of the paper. Dissolve the sample residue in the test tube with eight drops of acid acetone. Select a point on the line of origin at least two inches from the edge of

the paper, and apply successively 5 to 10 microliter (mcl) portions of the acid acetone solution until it has all been applied. After each application, dry the spot in a stream of air. At other points spaced one inch apart, apply a single 2 to 5 mcl portion of each of the standard solutions. With a spraying device, saturate the paper with the pH 3.5 buffer solution. Allow the paper to dry until just damp to the touch. Form a cylinder by bringing the outer edges of the paper together, allowing about one inch overlap and secure with a paper clip. Stand the paper in the chamber which has been filled to a depth of 3/4 inch with the resolving solvent.

(NOTE: PYRIDINE IS TOXIC. USE AN EXHAUST HOOD.)

After the solvent front rises to a height of 4 to 5 inches above the origin, remove the paper from the chamber and hang it up to air dry in the exhaust hood. After drying, hold the paper over a beaker containing a few drops of concentrated ammonia and then examine in a darkened room under long-wave UV light, (or use a UV light box). The tetracycline appears as yellow fluorescent spots. Identify the unknown sample by observing the position of the fluorescent spot(s) it has produced relative to positions of those produced by the standards, noting the position of the solvent front in relation to the position of the spots (RF values).

6.642 Thin Layer Chromatography (TLC)

Use a chromatographic jar with a rack with clips attached to suspend the sheet and a shallow tray in the bottom to contain the resolving solution. The sheet when placed in the chamber should dip into the solvent so that the level of the solution is below the origin line. Since silica gel is highly hygroscopic, store the sheets in a desiccating chamber after the package is opened. Saturate the sheet with the pH 3.5 buffer solution, and dry it in a hot air oven at 100° C. for 30-60 minutes. Allow the sheet to cool and place it over a lined template of heavy paper with dark lines spaced 1/4 inch apart. Keep a line 3/4 inch from the bottom edge of the TLC visible. This will be the line of origin. Attach the sheet to the template with paper clips to help maintain proper alignment. Spot the plate the same as the paper mentioned above. Place it in the chamber, suspending it by clips and letting it dip into the solvent solution. Allow the front of the resolving solution to rise 4 to 5 inches above the origin line. Remove the sheet from the chamber, air dry (under exhaust hood), and examine as with the paper chromatogram, described previously.

NOTE: Use ammonia to enhance weak reactions, but not for plates that are satisfactory without it.

By H. G. Fugate, Microbiologist

7.0 Animal Species Determination, Serological

7.1 Tube Precipitin Test (Presumptive)

7.11 Equipment and Materials

(1) Vials, glass, 6 x 50 mm, disposable.
(2) Pipettes, Pasteur type, nine inch, disposable.
(3) Pipettes, calibrated, assorted sizes.
(4) Vials, serum, rubber stoppered, 15 and 30 ml.
(5) Racks, vial holding, wooden. For 6 x 50 mm. vials.
(6) Tubes, culture, 20 x 150 or larger.
(7) Filter paper, Whatman #42, 11 cm. in diameter.
(8) Filter units, membrane type, microsyringe with Luer fitting.
(9) Filters, membrane type, 0.45 micron porosity, for item (8).
(10) Prefilters, fiberglass, for item (8).
(11) Syringers, disposable, assorted sizes.
(12) Needles, hypodermic, disposable, 20 and 22 gauge, one inch length.
(13) Centrifuge, preferably refrigerated.
(14) Tubes, centrifuge, plastic, autoclave, 50 ml. capacity.
(15) Colorimeter, Bausch & Lomb Spectronic 20, or similar.

Thoroughly clean all glassware used in direct contact with tissues or tissue products, distilled water rinse, and heat at least two hours at 200° C. in a drying oven to eliminate contamination from prior use.

7.12 Reagents

(1) Normal saline (0.85 percent sodium chloride solution). Dissolve 8.5 grams NaCl in 1000 ml. distilled water.
(2) 2X saline (1.7 percent sodium chloride solution). Dissolve 17 grams NaCl in 1000 ml. distilled water. Add merthiolate to a final concentration of 1:10,000.
(3) Normal Serums. Horse, beef, pork and sheep serums, for use as antigens in control tests.
(4) Antisera. Anti-horse, beef, pork, sheep, and any other species to be tested. (See 7.14).
(5) Biuret Solution. See 7.3.

7.13 Preparation of Antigen for Controls.

Obtain authentic serums from blood of known animals. Keep serums frozen until diluted for use.

The total protein content of serums varies from animal to animal within a species, as well as among various species. This makes it

necessary to adjust the level of antigen in controls. To avoid this difficulty, use the protein content as the basis for dilutions.

Determine the total protein (TP) content of the serum by the biuret method (Section 7.3). Prepare 1:500 dilution of TP using the following formula:

(5 x % TP) - 1 = D_V 500. In which % TP = percent total protein in serum, and D_V 500 = Volume of normal saline to be added to one volume of serum to attain a 1:500 dilution.

Examples:

Serum A = 7 percent TP	Serum B = 6.5 percent TP
(5 x 7) - 1 = 34	(5 x 6.5) - 1 = 31.5
1 ml. Serum A plus	1 ml. Serum B plus
34 ml. normal saline	31.5 ml. normal saline
equal 1:500 TP	equal 1:500 TP

Make further dilutions according to the following:

From	Take	Add saline (ml.)	Vol. (ml.)	Reciprocal of Dilution	Final Vol.
500 TP	10 ml.	20	30	1,500 TP	18 ml.
1,500 TP	10 ml.	10	20	3,000 TP	17 ml.
1,500 TP	2 ml.	18	20	15,000 TP	20 ml.
3,000 TP	3 ml.	27	30	30,000 TP	20 ml.
30,000 TP	10 ml.	10	20	60,000 TP	20 ml.

The 1500 and 3000 dilutions serve as heterologous antigens, and higher dilutions serve as homologous antigens in the tests that follow.

Filter diluted serums through a membrane filter (0.45 micron) into sterile vials or screw cap tubes. Store the diluted serums at 4 - 6° C. Do not freeze. Discard after three weeks, or if cloudy or precipitated.

7.14 Antiserum Production. Prepare antiserums effective against various animal species by the method of Proom (7.4), using the following departures from his method:

1. Adjust the pH of the solution to 6.2 - 6.3 instead of 6.5.

2. Wash the precipitate at least twice, perhaps three times, first shaking it on a mechanical shaker for thirty minutes and then centrifuging.

3. Instead of 5 ml., use 7.5 ml. injections into each leg (total 15 ml.).

4. On day 15, post injection, inject 5 ml. into each leg again. On day 23 - 25, bleed the rabbit.

5. To get the best serum volume, anesthetize the animal with carbon dioxide and bleed by cutting the throat. Then place the carcass head down into a stainless steel funnel and collect the blood in a large test tube.

6. Permit the blood to clot at room temperature for 2 to 4 hours; gently remove the clot from the glass walls and place lead weights on it. Refrigerate overnight, then decant the serum. Centrifuge it and add merthiolate to a concentration of 1:5,000. Filter the antiserum into sterile 30 ml. rubber stoppered serum vials (from which the air has been evacuated), using a membrane filtering apparatus (25 mm syringe adapter, 0.45 micron).

Refrigerate the antiserum until used. Do not freeze.

7.15 Antiserum Dilution Scheme for Titer and Specificity Tests

(a) Place five tubes (13 x 100 mm.) for each serum into a tube rack.

(b) Mark tubes as shown below:

Tube No.	0	1	2	3	4
Marking	1+0	1+1	1+2	1+3	1+4
2X saline (ml.) (7.12(2))	0.0	0.5	1.0	1.5	2.0
Antiserum (ml.)	1.0	0.5	0.5	0.5	0.5
Dilution	---	1:2	1:3	1:4	1:5

Filter-sterilize the diluted antiserums through membrane filters (0.45 microns) into sterile 15 ml. screw cap vials. Refrigerate at 4 to 6° C. DO NOT FREEZE. Discard within two weeks, or when control tests (7.16) show inadequate titer or specificity.

Test each of the diluted antiserums against the TP homologous dilutions (7.13) as described in 7.16, using the test method described in 7.18.

7.16 Control Tests. Choosing the Working Dilution. Prepare protocols to test all dilutions of antisera (7.15) against the homologous TP dilutions previously prepared (7.13), using the method described in 7.18. Choose for the test on the unknowns the highest dilution of antiserum that gives a positive test with the 30,000 TP within 8 minutes, and fails to give a positive test with the 1,500 TP (heterologous) within 10 minutes. This is the "Working dilution." Note exceptions: To test antibovine antiserum with heterologous sheep TP, use the 3,000 TP instead of the 1,500, and antiovine with heterologous bovine, use the 3,000 TP.

Each week, reconfirm the titer and specificity of the working dilutions of antiserums against appropriate TP dilutions.

7.17 Sample Preparation

7.171 Fresh Tissue. Weigh 10 to 25 g. of fresh tissue, using the inner portion of the piece if possible. Dice the tissue and place into an appropriate receptacle (polyethylene bag or beaker). Add 4 ml. normal saline (7.12(1)) for each gram of tissue. Allow to stand for 1-1/2 to 2 hours at room temperature. Filter 5 - 6 cc of the extract through three-fold filter paper (Whatman #42) into 20 x 150 mm. tubes. The filtrate must be crystal clear, but may be colored from straw to dark red. If the filtrate is not crystal clear, refilter. Run the test as soon as possible, before the filtrate becomes cloudy.

7.172 Partially Cooked or Cured Tissue. When a tissue has been heated above 165-175° F., the proteins become insoluble and cannot be extracted. Frequently, however, an interior section may not have reached the denaturing temperature and will release enough soluble proteins for a test. The same applies to cured products. For cooked, uncured, tissues, add 4 ml. saline (7.12(1)) for each gram of tissue, and let stand in the refrigerator at least 18 hours, then test aliquots at intervals for three days. If no reaction occurs after three days' extraction, report sample as not giving an antigenic response. Use the same procedure for cured tissue, but extract with distilled water instead of saline.

7.173 Chopped, Ground or Emulsified Tissue. Proceed as in 7.171 or 7.172, but use approximately 50 grams of tissue.

7.18 Precipitin Tube Test. In an appropriately marked rack, place one 6 x 50 mm. tube for each species for which the sample is to be tested (e.g. horse, beef, pork, sheep). Place in the tube, ca 0.1 ml. of the working dilution of antiserum (7.16), using a Pasteur pipette. Draw the tip of another Pasteur pipette to a fine point. Tilt the rack of tubes at a 45° angle and slide the pipette down the side of the tube of just above the antiserum. Then allow the extract

of the unknown (7.17) to flow gently over the surface of the antiserum, while withdrawing the pipette, keeping it ahead of the advancing interface. Do not allow the pipette to touch the antiserum, or to disturb the interface. Clean the surface of the tube with cheesecloth saturated with glycerin, then wipe it dry. After 3 to 5 minutes, and again up to 8 minutes, read the tube by indirect light against a black background.

A cloudy white ring at the interface is a positive test. Also test heterologous TP dilutions, and read up to 10 minutes as a test of acceptability of antisera (7.16). If the heterologous TP dilution for one species gives a positive test against the serum of another species within 10 minutes, check for possible contamination of the antiserum).

Retest the antiserum for specificity and retest the sample, extracting at least two times. If more than one piece of tissue was used, then retest each piece separately using, if possible, the innermost portions of the pieces. If the sample is ground or chopped, retest another extraction of the sample; repeat two times if the reaction indicates possible violation. Record the reaction times.

Confirm positive results, if desired, using the agar gel diffusion test (7.2).

7.2 Agar Gel Diffusion Test (Confirmation)

7.21 Equipment and Materials

(1) Dish, Petri, plastic, 15x100, disposable
(2) Pipettes, disposable, capillary, Pasteur type
(3) Box, plastic, humidity chamber, or other relatively air tight container used to maintain high humidity.
(4) Cutter, agar-gel, or template pattern
(5) Flask, side arm
(6) Tubing, rubber or neoprene, high vacuum type
(7) Tubing, brass, 5/32 inch x 1-3/4 inch (Cork borer)
(8) Applicators, wooden, cotton tipped
(9) Pipettes, graduated, serological, assorted sizes
(10) Dishes, staining (only if agar is to be dried and stained)
(11) Slides, microscope, 1 x 3 inch (only if agar is to be dried and stained)
(12) Filter paper, Whatman No. 1 and No. 42
(13) Pans, plastic, 6 x 12 x 6 inch, or other suitable containers (used only if agar is to be dried and stained)
(14) Assorted laboratory flasks, beakers, tubes, etc.

Clean all glassware, rinse in distilled water and heat a minimum of two hours at 200 C in a dry heat oven to eliminate contamination from prior use.

7.22 Reagents:

(1) Normal saline, (0.85 percent solium chloride solution): Dissolve 8.5 gm. NaCl in 1000 ml. distilled water.

(2) Buffered saline (0.85 percent sodium chloride solution, pH 7.2 phosphate buffered): To 1000 ml. normal saline, add 1.25 ml. stock phosphate buffer solution. Adjust pH to 7.2 if required.

(3) Phosphate buffer stock solution - pH 7.2: Dissolve 34 gm. monobasic potassium phosphate (KH_2PO_4) in 500 ml.. distilled water. Adjust pH to 7.2 with 1 normal sodium hydroxide (NaOH), (requires approximately 175 ml.). Dilute to 1000 ml. with distilled water. Store under refrigeration.

(4) Agar, 1.0 percent (Colab, Ion-Agar #2): To 99 ml. buffered saline, add 1.0 gm. #2 Ion-agar. Heat with constant stirring until agar is melted. Filter hot agar through glass wool or several thicknesses of cheese cloth. Dispense into screw cap flasks or tubes and sterilize by autoclaving for 20 minutes at 15 pounds pressure. Cool agar to 49-50 C and add 1.0 ml. of stock merthiolate solution (1:100), to give a final concentration of 1:10,000. Tighten caps (airtight) and store until needed. Remelt when needed in boiling water bath. (If caps are airtight, and no dessication or growth occurs, agar can be stored for extended periods of time).

(5) Tissue extracts from known animal species: Cut muscle tissue collected from animals (known species) into 10 gm. portions and freeze until needed. To 10 gm. of ground or finely diced tissue, add 30 ml. normal saline and mix. Let stand a minimum of 90 minutes. Decant liquid and filter through Whatman No. 42 filter paper. Use immediately.

(6) Antisera: Anti-, horse, beef, pork, sheep, and other species to be tested. (Section 7.14)

(7) Tissue extract - (unknown): Extract unknown tissue as in (5) above, using 20-25 gm. and 60-75 ml. normal saline.

(8) Staining solution: Dissolve 0.1 mg. Ponceau R in 10 ml. distilled water. Add 40 ml. of a mixture of 1.0 percent glacial acetic acid in 80 percent ethanol. Store in glass stoppered or rubber stoppered flasks/vials.

(9) Acid alcohol: To 99 ml. of 70 percent ethyl alcohol (ethanol), add 1.0 ml. glacial acetic acid.

(10) Acidified Distilled Water: To 1000 ml. distilled water, add 0.2 ml. glacial acetic acid.

(11) Mounting fluid: A commercially available material for mounting cover slips permanently.

7.23 Preparation of Agar-Gel Diffusion Plates:

7.231 Agar Plate Preparation. Remelt Ion-agar #2 (7.22(4)) and dispense 18-20 ml. into the 15x100 plastic petri dishes. Allow to solidify and refrigerate for a minimum of 30 minutes. Store no more than two weeks under refrigeration in a high humidity atmosphere. Do not use plates showing desiccation or microbial growth.

7.232 Cutting Pattern of Wells and Troughs. Remove the plates from refrigeration and cut the desired pattern by one of the two methods described below:

(1) Use a gel cutting tool which has the proper well and trough cutting tubes and knife edges permanently embedded in a fixture such as plexiglas or other solid substance. Figure 1 illustrates one such cutting tool. Align the tool carefully on the agar surface to obtain a perpendicular cut, then press down firmly to cut the agar.

(2) Using a pattern of the desired arrangement drawn on graph paper, center the plate over the pattern, agar side up. Press a metal tube of acceptable diameter, connected to a vacuum source by a vacuum tube and side arm flask through the agar at the indicated places on the pattern. Then cut the troughs with a razor blade or scalpel along the lines of the pattern; or use a tool fashioned with two blades or knife edges the correct distance apart, and with a downward motion cut the agar.

Remove the agar plugs in the wells with a metal tube connected to a vacuum source. Experience will dictate how to avoid tearing the agar surrounding the wells. Remove the trough plugs with an applicator stick which has one end shaved to present a shovel edge. Gently push the applicator stick to the dish bottom and guide it along the cut, raising the strip of agar as a plow would.

Remove the remaining agar in the wells and troughs with a cotton tipped applicator.

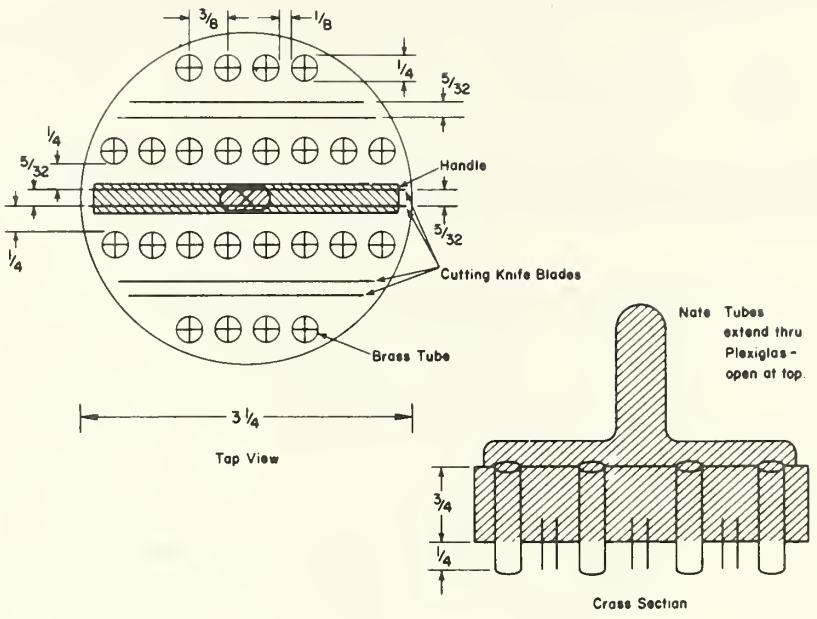

FIG. 1 -- Cutting tool used to cut pattern of wells and troughs in agar-gel. (Fugate and Penn, 1971)

7.233 Sealing Wells and Troughs

Hold the plate at a 45° angle and, with a Pasteur pipette, place a thin layer of agar on the floor of each well and trough, sealing the bottom edges of the cut agar to the plate. Do not add an excess of agar. Repair torn wells in troughs in a similar way; if necessary, refill the well or trough and recut it. Caution: An overfilled well will distort the agar and the reaction bands.

7.24 Preparation of Tissue Extracts: (Protein antigens) Using the desired known animal species muscle tissue, follow instructions as given in section 7.22(5). Do the same for tissue to be analyzed.

7.25 Charging Plates with Reactants: Mark the outside of the plates to identify the location of antisera and known and unknown extracts.

7.251 Charging the Wells: Using a Pasteur capillary pipette, partially fill the wells with the known and unknown extracts, maintaining a concave meniscus. Overfilling to form a convex meniscus will interfere with diffusion and may cause wells to overflow. Always place the extract of the unknown between known antigens of two different species. Like antigens will form continuous reactant bands in the agar media, and unlike antigens will form discontinuous bands (See Figure 2).

7.252 Charging of Troughs: Fill troughs with the antisera. Use one plate to determine two species only. (eg, beef and sheep, or beef and horse, etc.). Use the top and bottom troughs for one antiserum, and the center trough for the other. (See Figure 2).

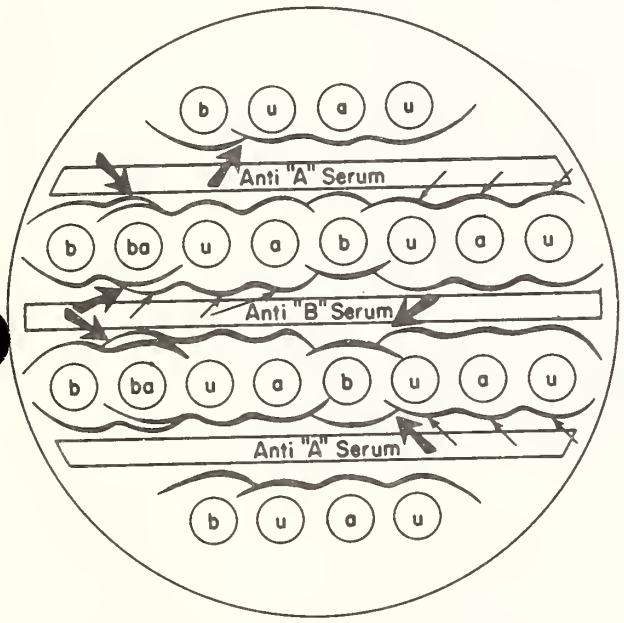

FIG. 2 — Precipitin pattern resulting from heterologous antigen-antisera reactions: a, antigens derived from species A; b, antigens derived from species B; u, antigens derived from unknown; ⟶. lines of partial identity; ⟶, lines of identity. Although atypical, the above pattern results when all antigens react with antisera used. The identification of unknown antigen u is accomplished by lines of identity formed with antigen a. Both a and u form lines of partial identity with lines formed by antigen b, which is indicated by a spur reaction. It can be concluded that antigen u is derived from species A and is similar but not identical to species B. (Fugate and Penn, 1971)

7.26 Incubation and Observation: Replace the plate covers and allow the plates to remain at room temperature for 1 1/2 to 2 hours. Refill the wells and troughs with the appropriate antigens and antisera. Line the bottom of an airtight chamber with wet filter paper or cotton. Incubate the plates in this high humidity chamber at room temperature for 18 to 24 hours. To read plates, direct a light source parallel to the agar surface, i.e., from the side of the plate, and hold the plate over a dark black background. The reactant bands will appear white on a grey surface. If the bands are not fully developed, refill the wells and troughs, and continue incubation in the chamber for 24 to 48 hours more, this time under refrigeration.

Following incubation, remove the plates from the humidity chamber, discard the remaining reactants and gently wash the plates under a stream of distilled water. Use soft cotton applicator to remove any film from the agar surface and precipitated matter from the wells and troughs. Dry the bottom of the petri dish with a soft laboratory tissue and observe the plate for reaction bands. Position the plate in alignment with the worksheet (Figure 3) and draw the reaction bands observed on the plate onto the worksheet.

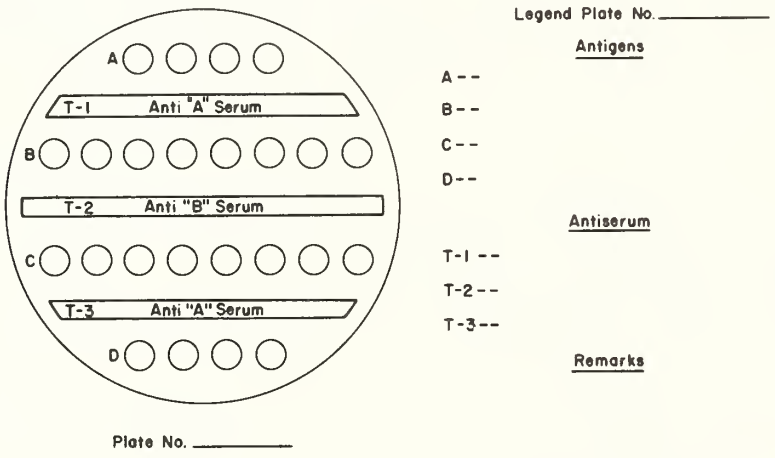

FIG. 3 -- Worksheet showing well and trough arrangement and antigen-antisera placement. (Fugate and Penn, 1971)

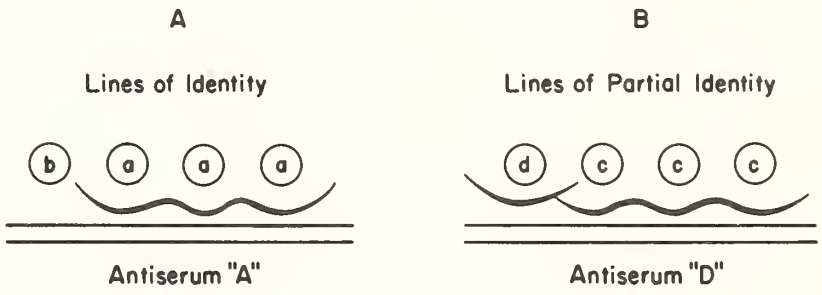

FIG. 4 -- Precipitin lines of identity and partial identity. A, lines of identity formed with homologous antigen-antiserum only (antigen a vs. antiserum A); B, lines of partial identity formed when similar antigens react with the same antiserum. Note the typical spur formed, indicating lines of partial identity. (Antigens c and d are similar but not identical). (Fugate and Penn, 1971)

7.27 Interpretation of Precipitin Reactions

Interpretation of results depends upon lines formed with known and unknown antigens. Figure 4 (A) illustrates an identity line, i.e., the precipitin line that forms when the antigens are identical. Figure 4 (B) shows partial identity lines, i.e., the lines that form when extracts contain similar but not identical proteins which react with the same antiserum. Figure 2 illustrates a typical reaction with an unknown and 2 known antigens, showing lines of identity and partial identity. Since unknown antigen u forms a continuous wave pattern with known antigen a, lines of identity form. The lines formed by known antigen b appear as spurs of those formed by antigen a and u, and are typical lines of partial identity.

Figure 2 also illustrates the pattern of precipitin lines formed when the sample contains tissue antigens from 2 species (wells ba). In the majority of cases, the antisera will not react with heterologous antigens and lines of partial identity do not form. This occurs when the animal species are closely related (such as bovine and ovine).

Figure 5 illustrates areas containing identical antigen alignment. Four of the 6 areas have antigens reacting with antiserum A and 2 of the 4 areas are in position to react with antiserum B. The 2 remaining areas (2 and 4) are control as well as indicative sites. The mixtures of antigens a and b in wells marked ba are in position to react with both antisera and illustrate precipitin lines that occur when the sample contains tissues from both species.

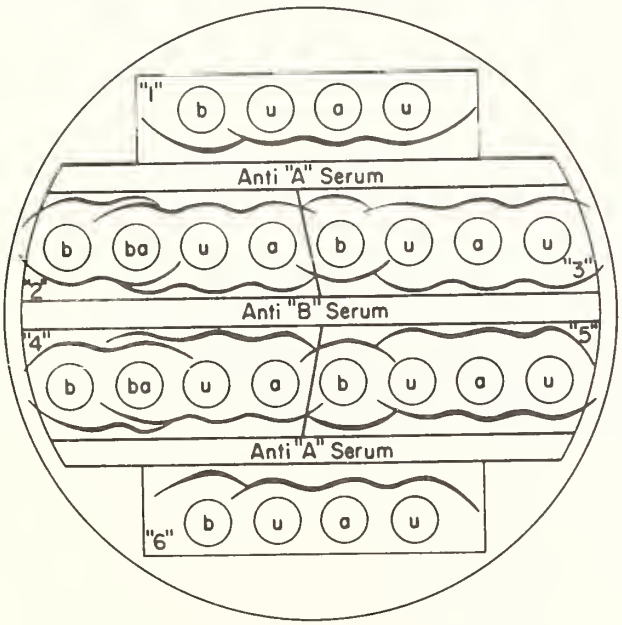

FIG. 5 -- Position and reaction sites (6 areas) each consisting of 4 antigen wells.
With the exception of areas 2 and 4, antigen placement is identical in each area. Areas 2 and 4 utilize one well each for a mixture of the 2 known antigens (ba), and illustrate precipitin reactions when sample consists of tissues from both species. All areas, except 1 and 6, are positioned to react with both antisera. Interpretation of results from areas 1, 3, 5, and 6 should correlate. Lines enclosing areas indicate portion of plate mounted on slides for preservation.
(Fugate and Penn, 1971)

7.28 Staining Reaction Bands

To keep a permanent record, dialyze to remove free proteins and salts, then dry, stain, and prepare a mount under a cover slip, as follows:

Flood the plate with 500 to 1000 ml. pH 7.2 buffered saline (7.22(2)) in a plastic pan (7.21(13)). Replace with fresh buffer twice daily for three days, then once daily for two more days. Finally replace with acidified distilled water (7.22(10)) and let stand overnight.

Drain off the acidified distilled water, and cut a block of the reaction area from the agar, and place it onto a 1 x 3 inch marked glass slide. Cover the block with a strip of filter paper, and dry in the incubator to a very thin film. Wash gently with a cotton applicator wetted with distilled water to remove adhering bits of the filter paper. Stain the films in Ponceau R taining solution (7.22(8)) for 10-15 minutes. Decolorize using acid-alcohol (7.22(9)) until agar is clear. Allow slides to dry, then mount under cover slips with mounting fluid (7.22(11)).

7.3 Total Protein by Biuret Method

7.31 Biuret Solution

In a liter flask place 1.5 gm. copper sulfate, and 6.0 gm. fine crystals sodium potassium tartrate. Add sufficient distilled water to dissolve. Add slowly, and with agitation of the flask, 300 ml. 2.5N sodium hydroxide and mix. Add 1 gm. potassium iodide and shake until dissolved. Dilute to volume. Discard when black or reddish precipitate forms.

7.32 Method

(1) Place 9.5 ml. 0.85% NaCl in a test tube. Add 0.5 ml. of sample. Rinse out pipette by drawing in and expelling some of the mixture.
(2) Into one of 2 test tubes place 2 ml. of the diluted sample, above; in the other, 2 ml. 0.85% NaCl solution (blank).
(3) Add 8 ml. biuret reagent (above) to each tube, and mix.
(4) Set 100% transmission with "blank" at wavelength 540 mmu.
(5) Immediately after adding biuret reagent read transmission of sample and obtain concentration from table.

7.33 Percent protein, as determined by percent transmission, Biuret reaction.

Percent Transmission, 540 mmu.	0	1	2	3	4	5	6	7	8	9
	Percent Protein									
0										
10										
20										
30								13.8	13.4	13.0
40	12.7	12.4	12.0	11.7	11.4	11.1	10.8	10.5	10.2	9.9
50	9.6	9.3	9.0	8.8	8.6	8.3	8.0	7.8	7.6	7.3
60	7.1	6.9	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.2
70	5.0	4.8	4.6	4.4	4.2	4.0	3.8	3.7	3.5	3.3
80	3.1	2.9	2.8	2.6	2.4	2.3	2.1	2.0	1.8	1.6
90	1.5	1.4	1.2	1.0						

*Example: Percent transmission = 47. Concentration of protein = 10.5%

7.4 REFERENCES

1. Proom, H., 1943. The Preparation of Precipitating Sera For The Identification of Animal Species. J. of Path. and Bact. 55, 419-426.

2. Fugate, H. Guy, and Penn, Shelton R. 1971 Immunodiffusion Technique For The Identification of Animal Species. J. of The Association of Official Analytical Chemists. 54, 1152-1156.

By Bernard Schwab, Senior Staff Officer

8. ANIMAL DISEASE MICROORGANISMS

8.1 General

8.11 Introduction

The methods described will recover a wide variety of pathogenic bacteria and permit their isolation and subsequent identification using routine diagnostic procedures. The recovery of significant organisms from diagnostic samples is dependent on a variety of factors, over some of which the diagnostician has no control. Some bacteria are found only in certain tissues and/or fluids of the host. It is wasted effort to try to isolate these pathogens from other than the proper specimen. However, when antemortem findings are inconclusive, field personnel are justified in sampling other tissues and the laboratory should cooperate. Adequate case histories also contribute to good laboratory diagnosis.

Any microbiologist with access to the proper equipment, media, antisera, reagents, stains, diagnostic keys and reference books should be able to identify most pathogenic bacteria. Some organisms require special media, anaerobic and/or carbon dioxide atmospheres, special incubation temperatures and extended incubation periods.

The samples may contain growth retarding agents such as antibiotics, or naturally occurring inhibitory immunological substances. Sometimes special steps to inactivate these agents will allow the viable organisms present to recover and grow.

8.12 Laboratory Safety

Any sample, regardless of origin or test request, may contain such organisms as Brucella, Mycobacterium, or other virulent agents. Handle all samples with care. Decontaminate all shipping containers properly prior to reissue. Instruct all sample-receiving employees in the proper handling of samples received for diagnostic microbiology. Even with conscientious safety practices a laboratory infection is possible. A momentary lapse in technique may disseminate a pathogenic agent in the laboratory environment, posing a potential threat to the microbiologists, other laboratory personnel, their families and the community. Certain pathogens in low number can produce disease in a healthy host.

The young, the debilitated, and aged are even more susceptible. Use biological safety hoods and maintain them. The hood is a supplement to but not a substitute for good microbiological technique. Blend or grind samples in the safety hood. Wear safety glasses routinely.

8.12 (Con.)

Routinely sterilize all materials before they leave the diagnostic laboratory. This includes all liquids before disposal into sewers, all solid waste, all laundry and equipment whether disposable or reusable. Practice good internal laboratory hygiene. Keep the laboratory in good order, and wash work areas routinely with 3% saponified cresol. Do not depend on ultraviolet light. Change clothes when entering or leaving the laboratory area. Wash hands frequently while working. Attend to laboratory accidents promptly. Smoke or eat in safe areas--never at the laboratory bench.

8.13 Equipment and Materials

(1) Incubators
(2) Refrigerator
(3) Freezer
(4) Centrifuge
(5) Biological Safety Hood and accessories
(6) Water Bath
(7) Autoclave
(8) pH Meter
(9) Microscope with oil immersion lens, and light source.
(10) Microscope slides, cover slips, immersion oil.
(11) Osterizers * (or other blender that will accommodate a Mason jar.)
(12) Sterile semi-micro blenders.
(13) Sterile Mason* jars, pint and quart sizes with lids.
(14) Sterile cutting assemblies and adapters for use with Mason* jars.
(15) Harvard trip balance.*
(16) Balance, 10,000 gm. capacity.
(17) Vortex* mixer.
(18) Electric soldering irons-600 watts, 110-120 volts.
(19) Anaerobic jars, catalysts, and indicators.
(20) Disposable hydrogen and CO_2 generator envelopes.

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* Use of trade names is for identification only and does not constitute endorsement by the Scientific Service Staffs or by the U.S. Department of Agriculture. In most instances, other brands of equipment similar to named items are satisfactory.

8.13 (Con. #2)

(21) Disposable CO_2 generator envelopes.
(22) Electric staining rack.
(23) Pipettes, assorted sizes.
(24) Pasteur pipettes and rubber bulbs.
(25) Knives, assorted sizes.
(26) Scalpel handles and assorted sizes of disposable blades.
(27) Scissors, surgical.
(28) Swabs, cotton tip, sterile.
(29) Forceps
(30) Whirl-Pak* bags.
(31) Plastic bags, assorted sizes, sterile.
(32) Paper pie plates, sterile.
(33) Tubes, centrifuge, plastic 50 ml., sterile.
(34) Racks for 50 ml. tubes.
(35) Electric shaker.
(36) Timers
(37) Stainless steel canisters with lids, assorted sizes.
(38) Instrument trays with lids.
(39) Stainless steel pans, 10" x 16" x 2".
(40) Aluminum trays 16" x 26" x 4".
(41) Agglutination slides, glass, with ceramic rings.
(42) Wood applicators.
(43) Wire letter baskets.
(44) Gloves, rubber or vinyl, disposable.
(45) Safety glasses.
(46) Test tube racks, assorted sizes.
(47) Test tubes, assorted sizes.
(48) Erlenmeyer flasks.

8.14 Reagents

(1) Refer to 4.12 and 4.13 for enterics.
(2) Reagents for Nitrate reduction test.
(3) Butterfield's buffered phosphate diluent (20.1).
(4) Sodium hypochlorite 5.25% solution.
(5) Phosphoric acid, 10% solution.
(6) Hydrogen Peroxide 3% solution, 30% solution.
(7) Phenol red indicator 0.4%
(8) Andrade's indicator.
(9) Methylene blue indicator.
(10) Buffer solutions for adjusting pH meter.
(11) Oxidase test paper.
(12) Shigella antisera set.
(13) Proteus OXK, Proteus OX19, Proteus OX2 antisera.

8.14 (Con.)

(14) Brucella abortus antisera.
(15) Pasteurella tularensis antisera.
(16) Leptospira Pools 1, 2, 3, and 4 antisera.
(17) Salmonella polyvalent "O" and "H" antisera.
(18) Salmonella somatic antisera A-I.
(19) Bethesda ballerup polyvalent antisera.
(20) E. coli polyvalent A, polyvalent B, polyvalent C, polyvalent D, and polyvalent E antisera.
(21) Lancefield Streptococcus grouping antisera.
(22) Candida albicans antisera.
(23) Leptospira canicola, Leptospira pomona antigen.
(24) Proteus OXK, Proteus OX19, Proteus OX2 antigen.
(25) Pasteurella tularensis antigen.
(26) Brucella AMS polyvalent antisera and antigens.
(27) Sensitivity disks-Penicillin, Bacitracin, Kanamycin.
(28) Coagulase plasma EDTA.
(29) Pneumococci-Differentiation Disks.

8.2 The Examination of Tissues, Fluids, and Other Animal Products for Pathogenic Agents.

8.21 Sample Submission

Veterinarians or meat inspectors should submit samples following instruction in Manual of Meat Inspection Procedures, paragraph 318.97. Except in unusual cases, samples must be frozen on receipt and maintained frozen until analysed. In certain instances, samples are submitted in borate or chlorine solution. Analyse these samples, but report that samples were sent in this manner. (The use of borate solution or chlorine is not recommended as they have a selective action on bacteria. The laboratory diagnosis may not be accurate due to this factor).

8.22 Sample Types

A case usually includes one or more of the following:

(1) Tissues samples such as muscle, lung, liver, spleen, lymph node, kidney, brain and the like;
(2) Fluids such as blood, serum, urine, pus, exudates, synovial fluid, and the like; and
(3) Others such as lesions, antibiotic injection sites, wounds, skin, feces, and other animal products.

8.23 Preparation of Samples for Analysis

8.231 Thawing

Thaw frozen samples overnight in a refrigerator, or for four hours at room temperature without removing them from the sample bag or sample container. After thawing samples, remove sample from sample bag or sample container and transfer to a sterile pie plate or sterile metal pan. Leave liquid or semi-liquid samples in their original sample containers.

8.232 Visual Inspection

Examine tissue sample for evidence of disease processes such as lesions or abscesses. If evident, excise these areas with sterile instruments and transfer to a sterile container. Record any abnormalities observed. This may not always be possible with frozen samples.

8.233 Surface Decontamination Procedures and Further Sample Preparation

8.2331 General

Disregard instructions to decontaminate surfaces, if the person who took the sample used aseptic precautions, and the sample arrived well wrapped and frozen. Decontamination procedures are a poor substitute for aseptic precautions because they may not only selectively destroy the contaminants, but also the pathogen(s) being sought. Use methods described under 8.2333, paragraph 2 to analyse samples submitted in borate or chlorine solution.

Rinse badly soiled samples with sterile distilled water. As an alternative, pre-soak in a laundry enzymatic cleaner previously shown to be free of bacteria.

8.2332 Frozen Tissue Samples

If the specimen is at least one half inch thick and has no visible lesions or other surface evidence of disease, sear the surface and sides with a hot soldering iron. With sterile dissecting instruments aseptically remove from beneath the seared area several grams of sample. Transfer the aliquot to a sterile pint jar. For each gram of sample add 3 ml. of Brain Heart Infusion Broth (20.6) or Trypticase Soy Broth (20.39). Replace jar lid with sterilized Osterizer cutting assembly and adapter for use with Mason jar and tighten securely. Blend sample with Osterizer in biological safety hood for one to two minutes. Use this preparation as the inoculum.

8.2333 Small Tissue Samples

Do not sear tissue samples, such as lymph nodes, but prepare it first as described in 8.2331, paragraph 2. Then with sterile forceps transfer the small tissue sample to a sterile pie plate or metal pan. Excise and discard fat. If no visible lesions or disease processes are apparent, transfer the sample to a sterile pint jar. Add sufficient chlorine solution so that there will be a chlorine residual after the tissue has been immersed for 15-30 minutes. (200 to 750 ppm chlorine solution is routinely used by this laboratory). Intermittently, vigorously shake the pint jar containing the sample in the chlorine solution. After the sample has been exposed to the chlorine solution for 15 minutes remove the tissue with sterile forceps. Let excess solution drip off the sample into the pint jar. Place the sample on a sterile pie plate or metal pan. Cut up the tissue into small pieces with sterile instruments. Aseptically transfer the pieces with sterile forceps to a sterile preweighed Mason pint jar. Determine the weight of the sample. Add 3 ml. of Brain Heart Infusion Broth (20.6) or Trypticase Soy Broth (20.39) per gram of sample. Replace the pint jar lid with the sterilized Osterizer cutting assembly and adapter, and tighten securely. Blend the sample and diluent by tilting and rotating the pint jar attached to the blender motor assembly to catch the tissue pieces by the cutting blades. Use this preparation as the sample inoculum.

8.2334 Sample Under 5 Grams

Do not sear or chemically disinfect a sample this small. Rinse the sample thoroughly with sterile distilled water. Transfer the sample with sterile forceps to a sterile pie plate or petri dish and mince with sterile instruments or grind with a small amount of a nutritive broth (20.6) or (20.39) and sterile sand in a sterile mortar and pestle. Use minced or ground material as the inoculum. Use this method, also, to prepare samples suspected of containing strict anaerobes.

8.2335 Liquid and Semi-Liquid Samples

These samples when submitted for routine diagnostic microbiology receive no prior treatment before being used as the inoculum. Keep them in the container in which they are submitted and aseptically transfer the inoculum from the sample container to the culture media or slide.

8.2336 Lesions

Excise lesions with sterile instruments from the surrounding tissue and transfer to a sterile petri dish. Mince them and then transfer

them with a sterile swab or knife blade to a small sterile screw cap tube. Add a small quantity of a nutritive broth. Mix the tissue and broth thoroughly. Use this preparation as the sample inoculum.

8.24 Routine Sample Analysis

The method that follows applies to routine diagnostic samples. To recover a specific agent, use special methods. For example, for Salmonella, see Section 4.

8.241 Microscopy

Make a microscopic examination of the inoculum under analysis. With a sterile swab or loop transfer a part of the inoculum to a pre-flamed and cooled slide. Make a thin film. Perform the Gram stain (20.45). Examine the slide microscopically with the oil immersion lens. Microscopy will indicate types and numbers of microorganisms present. The slide represents only a minuscule part of the specimen, and some microorganisms present in low numbers, may not be observed. Record findings of microscopic examination.

8.242 Media Inoculation

Judge the size of the inoculum to use from the microscopic findings on the inoculum. Generally, inoculate fluid media with 0.5-1 ml. of sample inoculum per ten ml. of fluid media. Inoculate plates with sterile cotton swabs or sterile cooled loops depending on the number of bacteria present in the inoculum. Streak plates with a sterile cool loop so that individual colonies are readily visible.

8.243 Primary Inoculation Media

(1) Inoculate Fluid Thioglycollate Medium (20.44) or Modified Cooked Meat Medium (20.9).
(2) Inoculate a Blood Agar plate (20.43). Incubate broth and plate at 35-37°C. Incubate the blood plate under 5% CO_2 atmosphere.

8.244 Further Studies

(1) After 18 to 24 hours incubation, examine the blood plate for growth and colony type. Make smears of representative colonies and Gram stain (20.45). Examine the slides microscopically and record morphology and Gram stain reaction. If no growth occurs on the blood agar plate, reincubate for an additional 24-72 hours and reexamine as above.
(2) After 18 to 24 hours incubation, make smears of broth cultures, Gram stain, and examine microscopically. Record morphological types

and Gram stain reaction. If no growth occurs, reincubate broths for an additional 24 to 72 hours and examine as above.
(3) Correlate the microscopic results from the broth smear and the blood plate smear. If, for example, Gram positive cocci, Gram positive rods, and Gram negative rods grow in broth media, and only Gram positive cocci and Gram negative rods grow on the blood agar plate incubated under 5% CO_2, inoculate a blood agar plate from the broth and incubate this blood agar plate under anaerobic conditions.

8.8 Pure Culture Studies

Select well isolated colonies for pure culture studies. With a sterile cool needle pick from the middle of a representative colony and transfer to a propagating broth (20.6, 20.39 or 20.4). If anaerobic organisms are suspected, use an anaerobic growth medium (20.44, or 20.9). Incubate the subcultures at 35 to 37°C. until adequate growth has occurred. Check the purity of the subcultures microscopically. Use only pure cultures for further studies and identification tests.

8.81 Identification Procedures

This section offers only abbreviated diagnostic keys. For further information, see the references (8.9).

Category	See Table
Cocci	I
Gram positive rods	II
Gram negative rods	III, IV, V

8.82
Table 1

Some Differential Characteristics of the Cocci

GROUP	AEROBIC GROWTH	GRAM STAIN	CATALASE	COAGULASE	REFERENCE	REMARKS
Streptococci	+	+	-	-	1	
Staphylococci	+	+	+	+	1	Report coagulase-positive staphylococci
Micrococci	+	+	+	-	1	
Neisseria	+	-			1,8	
Anaerobic Cocci	-				5,7,9,12	

* Neisseria usually grows aerobically, but there are exceptions

8.83
Table 2

Some Differential Characteristics of the Gram Positive Rods

GROUP	AEROBIC GROWTH	CATALASE	OXIDASE	MOTILITY	SPORES	ACID-FAST STAIN	REFERENCE	REMARKS
Bacillus	+	+	-	(+)	+	-	4	B. anthracis is non-motile
Clostridium	(-)	-	-	(+)	+	-	5,9,12	C. perfringens is non-motile
Corynebacterium	+	(+)	-	-	-	-	3,4	C. pyogenes is catalase negative
Erysipelothrix	+	-	-	-	-	-	3,4	
Listeria	+	+	-	+	-	-	3,4	Motile at room temperature
Lactobacillus	(+)	-	-	-	-	-	4	
Actinomyces	(-)	-	-	-	-	-	2,7,8,5,12	Most grow anaerobically
Mycobacteria	+	(+)	-	-	-	+	10,11	
Anaerobes, non-sporulating	-						2,5,7,9,12	Nomenclature in state of flux

+ : positive - : negative ( ) : exceptions; sign indicates most common reaction

8.84
Table 3

Separation of Enterobacteriaceae From Other Gram Negative Rods

GROUP	AEROBIC GROWTH	CATALASE	OXIDASE	GLUCOSE	NITRATE	REMARKS
Enterobacteriaceae	+	+	-	Acid (gas)	+	See Table 4
Others, Aerobes	+	±	±	±	±	See Table 5
Others, Anaerobes	-					See Table 5

8.85
Table 4 Some Reactions of the Enterobacteriaceae*

GROUP	SLANT	BUTT	GAS	H_2S	UREA	INDOL	CITRATE	MOTILITY	LYSINE DECARBOXYLASE	REMARKS
Shigella	K	A	-	-	-	±	-	-	-	Human pathogen
Escherichia	A(or)K	A	±	-	-	+	-	±	±	
Salmonella	K	A	+	(+)	-	-	+	+	+	
S. typhi	K	A	-	(+)	-	-	-	(+)	+	Human pathogen
Arizona	K(or)A	A	+	+	-	-	+	+	+	
Citrobacter	K(or)A	A	+	+	(-)	-	+	+	-	
Klebsiella	A	A	+	-	(+)	(-)	+	-	+	
Enterobacter	(A)	A	+	-	(-)	-	(+)	+	+	
Serratia	K	A	(-)	-	(-)	-	+	+	+	Some have red pigment
Pectobacterium	K	A	-	-	(-)	(-)	(+)	(+)	-	Plant pathogen
Proteus	A(or)K	A	+	±	+	±	+	+	-	Phenylalanine positive
Providencia	K	A	+	-	-	+	+	+	-	Phenylalinine positive
Edwardsiella	K	A	+	+	-	+	-	+	+	

K: Alkaline
A: Acid
* See Reference 6

+: positive
-: negative
( ): exceptions; sign indicates most common reaction

Table 5 Some Differential Characteristics of Gram Negative Rods, not Enterobacteriaceae

GROUP	AEROBIC GROWTH	CATALASE	OXIDASE	GLUCOSE (ACID)	CITRATE	REFERENCE	REMARKS
Actinobacillus	+	(+)	(-)	+	-	4,8	
Pasteurella	+	+	(+)	+	-	4,8	Some penicillin sensitive
Aeromonas	+	+	+	+	(-)	2,4,8	
Vibrio	(+)	+	+	+	(-)	4,8	
Pseudomonas	+	+	+	(+)	+	2,4,8	Some pigmented
Chromobacterium	+	+	-	(+)	(+)	4,8	Pigmented
Flavobacterium	+	+	+	(+)	(-)	4,8	Yellow pigment
Brucella	(+)	+	(+)	-	-	4,8	Coccobacilli
Moraxella	+	(+)	+	-	-	4,8	Coccobacilli to diplobacilli chains
Alcaligenes	+	+	+	-	(+)	4,8	
Mima	+	+	(-)	-	(+)	8	
Herella	+	+	-	+	(+)	8	
Bordetella	+	+	+	-	+	4,8	B. bronchiseptica (rapid urea pos.)
Hemophilus	+	(-)	(-)	(+)	-	4,8	
Bacteriodes	-					5,7,12	
Fusobacterium	-					5,7,12	

+ : positive - : negative ( ): exceptions; sign indicates most common reaction

8.9 REFERENCES

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20. MEDIA AND REAGENTS

Ingredients and reagents used to prepare the following media may be the product of any manufacturer if comparative tests have shown that satisfactory results are obtained. Carbohydrates will be chemically pure and suitable for biological use; inorganic chemicals will be ACS reagent grade; and dyes must be certified by "Biological Stain Commission" for use in media.

For convenience, dehydrated media of any brand equivalent to the formulation may be used unless instructions indicate otherwise. Test each lot of prepared medium for sterility and growth-promoting qualities of suitable organisms. (e.g., inoculate media containing lactose with coliforms; Staphylococcus media with Staphylococcus, etc.).

Hydrogen ion concentration (pH) will be determined, using an electronic pH meter which is standardized against known buffers, prepared according to AOAC (latest edition). Adjust pH when necessary by adding sufficient 1 Normal sodium hydroxide or 1 Normal hydrochloric acid.

Unless otherwise indicated, sterilize media by steam under pressure at 121° C. (15 lb.) for 15 minutes.

20.1 BUTTERFIELD'S PHOSPHATE DILUENT.

(a) Stock solution: Dissolve 34 grams KH_2PO_4 in 500 ml. distilled water, adjust to pH 7.2 with ca. 175 ml. 1 normal NaOH, and dilute to 1 liter. Store under refrigeration.

(b) Diluent: Dilute 1.25 ml. stock solution (a) to 1 liter with distilled water. Prepare dilution blanks using this solution, dispensing a sufficient quantity to allow for losses due to sterilization by autoclaving.

20.2 PLATE COUNT AGAR (STANDARD METHODS AGAR)

Pancreatic digest of casein USP	5.0 g.
Yeast extract	2.5
Dextrose	1.0
Agar	15.0

Suspend ingredients in 1,000 ml. of cold distilled water. Heat to boiling until all ingredients are dissolved. Final pH 7.0 ± 0.1.

20.3 LAURYL SULFATE TRYPTOSE BROTH (LST BROTH)

Trypticase or tryptose	20.0 g.
NaCl	5.0
Lactose	5.0
K_2HPO_4	2.75
KH_2PO_4	2.75
Sodium lauryl sulfate	0.1
Distilled water	1,000 ml.

Dispense into test tubes containing inverted fermentation tubes 10 x 75 mm. Final pH 6.8 $\pm$ 0.1.

20.4 EC BROTH

Tryptose or trypticase	20.0 g.
Bacto bile salt #3 or bile salt mixture	1.5
Lactose	5.0
K_2HPO_4	4.0
KH_2PO_4	1.5
NaCl	5.0
Distilled water	1,000 ml.

Dispense into tubes with inverted 10 x 75 mm. fermentation tubes. Final pH 6.9 $\pm$ 0.1.

20.5 BAIRD-PARKER MEDIUM

Basal Medium

Tryptone	10 g.
Beef extract	5 g.
Yeast extract	1 g.
Sodium pyruvate	12 g.
Glycine	12 g.
Lithium chloride $6H_20$	5 g.
Agar	20 g.
Distilled water	950 ml.

Suspend ingredients in water. Heat to boiling to dissolve completely. Dispense 95 ml. portions in screw-capped bottles. Autoclave 15 minutes at 121° C. Final pH 6.8 - 7.2.
Complete medium. Add 5 ml. prewarmed (45-50° C.) Bacto EY tellurite enrichment to 95 ml. molten basal medium, which has been adjusted to 45-50°. Mix well (avoiding bubbles) and pour

15-18 ml. into sterile petri dishes. Plates of complete medium should be stored in refrigerator for no longer than 48 hours before use. Plates should be dried before use:

(a) In a laminar flow hood with lids removed and agar surface upward; or (b) in a forced air oven or incubator for 2 hours at 50° C., with lids on the agar surface upward; (c) or in an incubator for 4 hours at 35° C. with lids on and agar surface upward; or (d) on laboratory bench for 16-18 hours at room temperature with lids on and agar surface upward.

20.6 BRAIN HEART INFUSION BROTH (BHI BROTH)

Calf Brain (infusion from)	200.0 g.
Beef heart (infusion from)	250.0
Proteose peptone or gelysate	10.0
NaCl	5.0
Na_2HPO_4	2.5
Dextrose	2.0
Distilled water	1,000 ml.

Dispense into Wasserman tubes. Final pH 7.4 $\pm$ 0.1.

20.7 TRYPTICASE SOY BROTH 10% SODIUM CHLORIDE (TSB 10% NaCl)*

Sodium chloride	100.0 g.
Trypticase (pancreatic digest of casein)	17.0
Phytone (papaic digest of soya meal)	3.0
K_2HPO_4	2.5
Dextrose	2.5

To make from commercial TSB, add 95 g. NaCl to 30 g. of dry ingredients, and dissolve in 1,000 ml. distilled water. Dispense into tubes. Final pH 7.3 $\pm$ 0.1.

* Dehydrated prepared medium not available commercially.

20.9 MODIFIED COOKED MEAT MEDIUM

(a) Cooked Meat Medium (dehydrated prepared medium available commercially)

Beef Heart	454.0 g.
Proteose Peptone	20.0
Dextrose	2.0
Sodium Chloride	5.0

(b) Diluent (not available commercially)

Trypticase	10.0 g.
Sodium Thioglycollate	1.0
Soluble Starch	1.0
Dextrose	2.0
Neutral Red (1% aqueous)	5.0 ml.
Distilled Water	1,000 ml.

Adjust to pH 6.8.

Add ca. 1 gram of (a) and 15 ml. of (b) to tubes no smaller than 20 x 150 mm.

20.10 BROM CRESOL PURPLE DEXTROSE BROTH (BCP)*

Dextrose	10.0 g.
Beef Extract	3.0
Peptone	5.0
Brom cresol purple, 1.6% in alcohol	2 ml.
Distilled water	1,000 ml.

Adjust to pH 7.0
Dispense into tubes no smaller than 20 x 150.

* Dehydrated prepared medium not available commercially.

20.11 ANTIBIOTIC MEDIUM #1

Peptone	6 g.
Pancreatic Digest of Casein	4
Yeast Extract	3
Beef Extract	1.5
Glucose	1
Agar	15
Distilled Water	1,000 ml.

Boil to dissolve. Dispense in 100 ml. quantities. Final pH 6.5 - 6.6.

20.12 ANTIBIOTIC MEDIUM #2

Peptone	6 g.
Beef Extract	1.5
Yeast Extract	3
Agar	1,000 ml.

Boil gently 1-2 minutes to dissolve. Dispense in 100 ml. quantities and autoclave. When cooled but still liquid (60-65° C.), add sterile dextrose solution to a final concentration of 1 g/liter. Final pH 6.6.

20.13 ANTIBIOTIC MEDIUM #8

Peptone	6 g.
Beef Extract	1.5
Yeast Extract	3
Agar	15
Distilled Water	1,000 ml.

Boil gently 1-2 minutes to dissolve. Dispense in 100 ml. quantities. Final pH 5.7 - 5.9.

20.14 ANTIBIOTIC MEDIUM #5

Peptone	6 g.
Beef Extract	1.5
Yeast Extract	3
Agar	15
Distilled Water	1,000 ml.

Boil gently 1-2 minutes to dissolve. Dispense in 100 ml. quantities. Final pH 7.9 - 8.1.

20.15 LACTOSE BROTH (4.9(11))

Beef Extract	3 g.
Peptone	5
Lactose	5
Distilled Water	1,000 ml.

Dispense into test tubes with inverted fermentation tubes, or 225 ml. portions into bottles or flasks. Final pH 6.7.

20.16 TETRATHIONATE BROTH (KAUFFMANN, 4.9(27))

Proteose Peptone	5 g.
Bile Salts	1
Calcium Carbonate	10
Sodium Thiosulfate	30
Distilled Water	1,000 ml.

Dissolve and heat to boiling. Cool below 45° C. Add 20 ml. iodine solution. (Dissolve 5 g. potassium iodide in 5 ml. distilled water. Add 6 g. iodine crystals and shake to dissolve. Add 15 ml. distilled water.) Brilliant green may then be added (10 ml. of 1:1,000 solution per liter). Shake to mix. Do not heat after the addition of iodine. Dispense into sterile containers and use the day it is prepared. The basal medium without the iodine may be sterilized by autoclaving and stored indefinitely.

20.17 TT BROTH (HAJNA AND DAMON, 4.9 (4))

Yeast Extract	2 g.
Tryptose	18
Glucose	0.5
d-Mannitol	2.5
Sodium Deoxycholate	0.5
Sodium Chloride	5
Sodium Thiosulfate	38
Calcium Carbonate	25
Brilliant Green	0.01
Distilled Water	1,000 ml.

Dissolve and heat to boiling. Cool below 50° C. Add 40 ml. iodine solution. (Dissolve 8 g. potassium iodine in 20 ml. distilled water. Add 5 g. iodine crystals and shake to dissolve. Add distilled water to volume of 40 ml.). Shake to mix. Do not heat after the addition of iodine. Dispense into sterile containers and use the day it is prepared. The basal medium without the iodine may be sterilized by autoclaving and stored indefinitely.

20.18 SELENITE -CRYSTINE BROTH (NORTH AND BARTRAM, 4.9 (20))

Tryptone	5 g.
Lactose	4
Na_2HPO_4	10
Sodium Selenite	4
Cystine	0.01
Distilled Water	1,000 ml.

Heat to dissolve. Avoid excess heating. Do not autoclave. Dispense into sterile containers.

20.19 BRILLIANT GREEN AGAR (KAUFFMANN, 4.9 (3))

Yeast Extract	3 g.
Proteose Peptone #3 (or Polypeptone)	10
Sodium Chloride	5
Lactose	10
Saccharose	10
Phenol Red	0.08
Brilliant Green	0.0125
Agar	20
Distilled Water	1,000 ml.

Boil to dissolve. Dispense to bottles or flasks. Sterilize at 121° C. for 12 minutes. Final pH 6.9.

20.20 BRILLIANT GREEN SULFA AGAR (OSBORN AND STOKES, 4.9 (6))

Yeast Extract	3 g.
Polypeptone	10
Sodium Chloride	5
Lactose	10
Sucrose	10
Phenol Red	0.08
Agar	20
Sulfapyridine	1
Brilliant Green	0.0125
Distilled Water	1,000 ml.

Mix thoroughly and heat with frequent agitation to dissolve. Dispense in bottles or flasks and autoclave.

20.21 XL AGAR BASE (TAYLOR, 4.9(7))

Xylose	3.5 g.
l-lysine	5
Lactose	7.5
Sucrose	7.5
Sodium Chloride	5
Yeast Extract	3
Phenal Red	0.08
Agar	13.5
Distilled Water	1,000 ml.

For XL Agar, sterilize XL Agar Base for 10 minutes at 118° C. Cool to 55° C. Add 20 ml. of aqueous solution of 34% sodium thiosulfate and 4% ferric ammonium citrate. Pour into plates.

For XLD agar, sterilize XL Agar Base for 10 minutes at 118° C. Cool to 55° C. Add 20 ml. of aqueous solution of 34% sodium thiosulfate and 4% ferric ammonium citrate. Add 25 ml. of 10% aqueous sodium desoxycholate. Pour into plates.

20.22 SS (SALMONELLA - SHIGELLA) AGAR (4.9(8))

Beef Extract	5 g.
Proteose Peptone (or Polypeptone)	5
Lactose	10
Bile Salts	8.5
Sodium Citrate	8.5
Sodium Thiosulfate	8.5
Ferric Citrate	1
Agar	13.5
Brilliant Green	0.00033
Neutral Red	0.025
Distilled Water	1,000 ml.

Heat to boiling to dissolve. Do not autoclave. Pour to plates, and permit surfaces to dry with tilted covers ca 2 hours before streaking. Final pH 7.0.

20.23 BISMUTH SULFITE AGAR (WILSON AND BLAIR, 4.9(22))

Beef Extract	5 g.
Peptone	10
Dextrose	5
Disodium Phosphate	4
Ferrous Sulfate	0.3
Bismuth Sulfite Indicator	8
Agar	20
Brilliant Green	0.025
Distilled Water	1,000 ml.

Heat to boiling to dissolve. Do not autoclave. Pour plates and use promptly. Remelting of agar is not permissible. Poured plates may be stored in refrigerator for not more than 3 days. Final pH 7.7.

20.24 TRIPLE SUGAR IRON AGAR (TSI) (HAJAN, 4.9(9))

Beef Extract	3 g.
Yeast Extract	3
Peptone	15
Proteose Peptone	5
Lactose	10
Saccharose	10
Dextrose	1
Ferrous Sulfate	0.2
Sodium Chloride	5
Sodium Thiosulfate	0.3
Agar	12
Phenol Red	0.024
Distilled Water	1,000 ml.

Heat to boiling to dissolve. Dispense into tubes. After sterilization, slant to give a generous butt.

20.25 LYSINE IRON AGAR (EDWARDS AND FIFE, 4.9(10))

Peptone	5 g.
Yeast Extract	3
Glucose	1
l-lysine	10
Ferric Ammonium Citrate	0.5
Sodium Thiosulfate	0.04
Brom Cresol Purple	0.02
Agar	15
Distilled Water	1,000 ml.

Dispense in tubes and autoclave. Slant with deep butt and short slant.

20.26 MR-VP MEDIUM (4.9(11))

Polypeptone or Buffered Peptone	7.0 g.
Dextrose	5.0
K_2HPO_4	5.0
Distilled Water	1,000 ml.

Dispense into tubes. Final pH 6.9 $\pm$ 0.1.

20.27 TRYPTONE BROTH

Tryptone or Trypticase	10.0 g.
Distilled Water	1,000 ml.

Dispense into tubes.

20.28 SIMMONS CITRATE AGAR (4.9(12))

Magnesium Sulfate	0.2 g.
Monoammonium Phosphate	1
Dipotassium Phosphate	1
Sodium Citrate	2
Sodium Chloride	5
Agar	15
Brom-Thymol Blue	0.08
Distilled Water	1,000 ml.

Dissolve with heat. Dispense into test tubes as slants, or into bottles. Streak slants or plates from a colony or culture without introducing a carbon source with the inoculum.

20.29 PHENOL RED TARTRATE AGAR (JORDAN AND HARMAN, 4.9(13))

Peptone	10 g.
Sodium Potassium Tartrate	10
Sodium Chloride	5
Agar	15
Phenol Red	0.024
Distilled water	1,000 ml.

Dissolve with gentle heat. Dispense 4.5 ml. in 13 x 100 tubes. Autoclave for 12 minutes at 121° C. Cool tubes promptly in upright position with cold running water.

20.30 MOTILITY MEDIUM (EWING AND EDWARDS)(4.9(1))

Meat Extract	3 g.
Peptone	10
Sodium Chloride	5
Agar	4
Distilled Water	1,000 ml.

Adjust to pH 7.4. Dispense in tubes.

20.31 UREA AGAR (CHRISTENSEN)(4.9(14))

Agar	15 g.
Distilled Water	900 ml.

Autoclave and cool to 50° C. Add filtered urea base. Mix and distribute in sterile tubes.

Base

Urea	20 g.
Peptone	1
Sodium Chloride	5
Glucose	1
Monobasic Potassium Phosphate	2
Phenol Red (1:500 solution)	6 ml.
Distilled Water	100 ml.

Adjust to pH 6.8. Sterilize by filtration.

20.32 FERMENTATION BROTH BASE (EDWARDS, 4.9(1))

Peptone	10 g.
Meat Extract	3
Sodium Chloride	5
Andrade's Indicator	10 ml.
Distilled Water	1,000 ml.

Adjust to pH 7.2. Add 1% carbohydrate.* Tube with inverted insert tubes and sterilize at 121° C. 12 minutes. Remove from autoclave and cool in running water.

* Lactose, Sucrose, Maltose, Arabinose and Xylose should be sterilized by filtration and added to previously sterilized broth base. Glucose, salicin, dulcitol, adonitol, inositol, sorbitol, raffinose, rhamnose, and trehalose may be added to broth base before autoclaving.

20.33 DECARBOXYLOSE MEDIUM (MOELLER, 4.9(15), (1))

Peptone	5 g.
Meat Extract	5
Brom Cresol Purple (1.6%)	0.625 ml.
Cresol Red (0.2%)	2.5 ml.
Glucose	0.5 g.
Pyridoxal	5
Distilled Water	1,000 ml.

Adjust to pH 6. Divide in four portions. Tube one portion without addition of amino acid, for control purpose. To one of the remaining portions, add 1% l-lysine dihydrochloride, to another add 1% l-arginine hydrochloride and to the third portion, add 1% l-ornithine dihydrochloride. Readjust to pH 6, if necessary. Tube (3 ml. per 13 x 100 tube). Sterilize 121° 10 minutes. (If d, l-amino acids are used, use 2% concentration).

After inoculation, layer with sterile mineral oil. Examine daily for four days.

20.34 MALONATE BROTH (LEIFSON, 4.9(16): MODIFIED, 4.9(17))

Yeast Extract	1 g.
Ammonium Sulfate	2
Dipotassium Phosphate	0.6
Monopotassium Phosphate	0.4
Sodium Chloride	2
Sodium Malonate	3
Glucose	0.25
Bromthymol Blue	0.025
Distilled Water	1,000 ml.

Dispense into test tubes.

(Caution: The commercially prepared malonate broths require addition of yeast extract and glucose to meet the above formula.)

20.35 KCN BROTH (MOELLER, 4.9(18), (1))

Base

Proteose Peptone #3 (or Orthana Special Peptone)	3 g.
Disodium Phosphate	5.64
Monopotassium Phosphate	0.225
Sodium Chloride	5
Distilled Water	1,000 ml.

Sterilize in the autoclave in 100 ml. amounts. Cool to below 20° C. Prepare 0.5% KCN with cold sterile distilled water. Using a sterile syringe or bulb pipet, add 1.5 ml. KCN solution to each 100 ml. of base. Distribute 1 to 2 ml. to small sterile tubes and stopper quickly with corks sterilized by heating in paraffin. Store the finished medium in the refrigerator not more than two weeks. CAUTION: KCN is a deadly poison.

20.36 PHENYLALANINE AGAR (EWING, 4.9(17))

Yeast Extract	3	g.
Dipotassium Phosphate	1	
Sodium Chloride	5	
l-phenylalanine*	1	
Agar	12	
Distilled Water	1	liter
*or d, l-phenylalanine	2	g.

Tube. Sterilize 121° C. 15 minutes. Slant.

20.37 NUTRIENT GELATIN

Beef Extract	3	g.
Peptone	5	
Gelatine	120	
Distilled Water	1,000	ml.

Autoclave 121° C. for 12 minutes. Cool promptly with cold running water.

20.38 V-P REAGENT OF O'MEARA, MODIFIED

Potassium Hydroxide	40	g.
Creatine	0.3	
Distilled Water	100	ml.

Dissolve alkali in water. Add creatine. Keep refrigerated. Make new reagent every 3 weeks.

Use equal parts of reagent and culture. Aerate by shaking. Place test at 37° C. Read in 4 hours.

20.39 TRYPTICASE SOY BROTH

Trypticase	17.0	g.
Phytone	3.0	
Sodium Chloride	5.0	
Dipotassium Phosphate	2.5	
Dextrose	2.5	
Distilled Water	1	liter

Dispense into tubes.

20.40 TRYPTOSE BROTH

Tryptose	20	g.
Sodium Chloride	5	
Dextrose	1	
Thiamine Hydrochloride	0.005	
Distilled Water	1	liter

20.41 VEAL INFUSION BROTH

Veal, infusion from	500	g.
Peptone	10	
Distilled Water	1	liter

20.42 LACTOSE BROTH-10X

Beef extract	30	g.
Peptone	50	
Lactose	50	
Distilled Water	1,000	ml.

Dispense appropriate amounts into tubes or flasks.

20.43 BLOOD AGAR BASE (COLUMBIA)

Pantone	10	g.
Bitone	10	
Tryptic Digest of Beef Heart	3	
Corn Starch	1	
Sodium Chloride	5	
Agar	15	
Distilled Water	1,000	ml.

Autoclave 121° C. for 15 minutes. Cool to 50° C. Add 5% sterile defibrinated sheep blood. Swirl. Avoid bubble formation. Pour 15 to 20 ml. into sterile petri dish.

20.44 FLUID THIOGLYCOLLATE MEDIUM

Pancreatic Digest of Casein (Trypticase)	15.0	g.
l-Cystine	0.5	
Dextrose	5.0	
Yeast Extract	5.0	
Sodium Chloride	2.5	

Sodium Thioglycollate	0.5
Resazurin	0.001
Agar	0.75
Distilled Water	1,000 ml.

Distribute 15 ml. per tube. Autoclave 121° C. for 15 minutes. Tighten caps. Store in dark in cool place. (Do not refrigerate).

20.45 GRAM STAIN (HUCKER MODIFICATION)

Reagents:

(a) Stock crystal violet:

Crystal violet, 85% dye	20 gm.
Ethanol, 95%	100 ml.

(b) Stock oxalate solution:

Ammonium oxalate	1 gm.
Distilled water	100 ml.

Working solution= dilute the stock crystal violet solution 1:10 with distilled water and mix with 4 volumes of the stock oxalate solution. Store in a glass-stoppered bottle.

(c) Gram iodine solution:

Iodine crystals	1 gm.
Potassium iodide	2 gm.

Dissolve potassium iodide completely in 5 ml. of distilled water; dissolve the iodide crystals; then add:

Distilled water	240 ml.
Sodium bicarbonate 5% aqueous solution	60 ml.

Mix well; store in an amber glass bottle.

(d) Decolorizer;

Ethanol, 95%	250 ml.
Acetone	250 ml.

Mix; store in glass-stoppered bottle.

(e) Counterstain: Stock safranin

Safranin	2.5 gm.
Ethanol, 95%	100 ml.

Working solution--dilute stock safranin 1:5 or 1:10 with distilled water; store in a glass-stoppered bottle.

20.46 GN BROTH (HAJNA)

Polypeptone	20.0	g.
Dextrose	1.0	
D-Mannitol	2.0	
Sodium Citrate	5.0	
Sodium Desoxycholate	0.5	
Dipotassium Phosphate	4.0	
Monopotassium Phosphate	1.5	
Sodium Chloride	5.0	
Distilled Water	1,000.0	ml.

Autoclave at 116° C. for 15 minutes or steam for 30 minutes at 100° C.

20.47 NEOMYCIN ASSAY AGAR (ANTIBIOTIC MEDIUM #11)

Gelsate Peptone	6.0
Trypticase Peptone	4.0
Yeast Extract	3.0
Beef Extract	1.5
Dextrose	1.0
Agar	15.0
Distilled Water	1,000.0 ml.

Autoclave 121° C. for 15 minutes. Refrigerate.

20.48 MODIFIED PE-2 MEDIUM

Peptone		20.0	g.
Yeast extract		3.0	g.
Bromcresol purple	2% in alcohol	2.0	ml.
Distilled Water		1,000	ml.

Dissolve with gentle heat if necessary. Dispense 19 ml. portions into 20 x 150mm screw-cap test tubes containing 8-10 untreated Alaska seed peas (Rogers Brothers Co., Seed Div., P.O. Box 2188, Idaho Falls, Idaho 83401, No. 423; Northurp King Seed Co., 1500 N.E. Jackson Street, Minneapolis, Minnesota 55413; or hardware store). Autoclave 30 minutes at 121° C. If not freshly prepared, heat to 100° C. for ten minutes and cool to 55° C. before using.

20.49 APT AGAR

Yeast Extract	7.5 g.
Tryptone	12.5
Dextrose	10.0
Sodium Citrate	5.0
Thiamine Hydrochloride	0.0001
Sodium Chloride	5.0
Dipotassium Phosphate	5.0
Manganese Chloride	0.14
Magnesium Sulfate	0.8
Ferrous Sulfate	0.04
Tween 80	0.2
Agar	15.0
Distilled Water	1,000 ml.

Dissolve and heat to boiling. Avoid excessive heating.

20.50 KF BROTH

Polypeptone or Proteose Peptone No. 3	10.0 g.
Yeast Extract	10.0
Sodium Chloride	5.0
Sodium Glycerol Phosphate	10.0
Maltose	20.0
Lactose	1.0
Sodium Azide	0.4
Brom Cresol Purple	0.015 g. or Phenol red 0.018 g.
Distilled Water	1,000 ml.

Dissolve and heat to boiling.

Before sterilization adjust the pH to 7.6 with a 10% solution of sodium carbonate (add approximately 0.6 ml/liter). Autoclave in 10 ml. amounts for 10 minutes at 15 pounds pressure (121° C.). Final pH should be 7.2.

20.51 TRYPTICASE SOY AGAR

Trypticase	15 g.
Phytone	5 g.
Sodium Chloride	5 g.
Agar	15 g.
Distilled Water	1,000 ml.

Suspend ingredients in water and heat to boiling with stirring. Sterilize at 121° C.

20.52 KOVAC'S REAGENT

Pure Amyl or Isoamyl Alcohol	150	ml.
Paradimethylaminobenzaldehyde	10	g.
Concentrated HCl	50	ml.

Dissolve aldehyde in alcohol and slowly add acid. The dry aldehyde should be light in color. Prepare reagent in small quantities. Store in refrigerator.

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